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VITAMIN B_1 CONTENT OF HUMAN MILK AS AFFECTED BY INGESTION OF THIAMIN CHLORIDE [1]

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(Received for publication April 17, 1939)

Much interest attaches to the question of the control of the composition of human as well as of cow's milk. If by suitable feeding desirable changes can be brought about particularly in the vitamin content of milk obvious advantages would result. Most of the efforts made to increase the vitamin content of cow's milk by feeding the cows vitamin-rich feeds have met with somewhat disappointing results, either very little increase being produced as in the cases of the water soluble vitamins or only small transferrals of the excesses fed as in the cases of vitamins A and D. Physiological control of the levels of both vitamins B_1 and C has been suggested as explaining the limited response of the milk vitamin content to forced vitamin feeding. Gunderson and Steenbock ('32) advanced this suggestion to explain lack of vitamin B increase in cow's and goat's milk following an increase in this vitamin in the ration and Hunt and Krauss ('31) came to the same conclusion after similar experiments on cows. Hess, Light, Frey and Gross ('32) administered vitamin B-rich irradiated yeast to cows and obtained no effect on the vitamin B_1 of their milk.

Less work has been done on the effect of vitamin feeding on human milk. McCosh, Macy and Hunscher ('31) determined

[1] The authors acknowledge with gratitude the assistance of Elsie Parks, Ruth Eichar, Doris Koch and Grace Kanzaki in the biological assays. Valuable assistance was also received from W.P.A. project 8877-A24 assigned to the University of California.

the vitamin B_1 content of the milk of three women before and during administration of 10 gm. dried yeast daily. These women were producing rather extraordinary amounts of milk, 1.5 to 3 liters daily. The yeast did not affect the volume of milk nor was there any clear indication of an increase in its vitamin B_1 content although some other fraction of the B complex seemed improved. Donelson and Macy ('34) also found that the administration of 10 gm. of dried yeast daily had no effect upon the vitamin B_1 content of the mixed milk of women producing large volumes of milk. The milks had rather uniformly 0.1 'Sherman' unit of vitamin B per cubic centimeter. The vitamin G (probably riboflavin) of the yeast milk was definitely increased from 0.2 to 0.3 Sherman unit per cubic centimeter. Neuweiler ('38) gave thiamin by intravenous injection and by mouth to three lactating women and produced temporary increases in the vitamin B_1 of their milk. He used the thiochrome method of assay and found in this way 5 to 13 μg. per 100 ml. in human milk and 21 to 57 μg. per 100 ml. in cow's milk.

Two young women, one in the early and the other in a later phase of lactation agreed to act as subjects for this experiment. Subject A made a weighed record of her food intake during the first month of the study and both she and subject B kept a careful diary in terms of measures of all food eaten throughout the experiment. The probable vitamin B_1 content of these food intakes was estimated using the best available compilations of assays (Williams and Spies, '38). The quantity of milk produced was estimated in the case of subject A by weighing the infant before and after each nursing but this record was not made regularly by subject B. Both infants were healthy and growing normally at all times and both mothers remained well and made no change in body weight.

Subject B had for several years been troubled with a mucous colitis and had consequently taken a smooth diet which was probably rather low in vitamin B_1. Subject A on the other hand was a student of nutrition and since she was particularly conscious of possible vitamin B_1 deficiencies in her diet had

taken pains to include therein whole cereals, wheat germ and yeast during both her pregnancy and lactation.

A sample of breast milk was obtained once a day by the subjects using a hand pump and these samples were brought at once to the laboratory where they were kept frozen solid until they were used.

The method of assay was that of rat growth after preliminary depletion. Autoclaved yeast was used as source of the B_2 complex. Crystalline thiamin chloride or the International clay adsorbate or both were used as standards fed parallel

TABLE 1

Vitamin B_1 of the diet and human milk production

SUBJECT	PERIOD	MONTH OF LACTATION	SUPPLEMENT THIAMIN CHLORIDE	VITAMIN B_1 IN THE DAILY DIET		VITAMIN B_1 IN THE MILK	TOTAL MILK PRODUCED	GAIN IN WEIGHT OF INFANT
			Mg. per day	Micrograms thiamin	Int. units	Micrograms per 100 ml.	Liters per day	Ounces per week
A	1	5th	None	1650	550	32	0.84	3.2
	2	6th	5	1150	385	..	0.54	4.8
	3	7th	10	1250	418	25	0.50	4.0
B	1	3rd	None	1050	350	11	0.60	4.0
	2	4th	5	1140	380	20	(estimated)	4.8
	3	5th	14.2	1060	355	25	(estimated)	8.0

with the milk to groups of litter mate rats. The period of assay was 28 days, and the breast milk samples were fed within a day or two of the date of collection. Raw and pasteurized market cow's milk was also fed in several cases as a further test of the reliability of the assays.

The periods used are indicated in table 1. The decreasing milk production recorded for subject A was due to the gradual weaning of the infant. No actual measurement of milk volume was made for subject B, but the amount obtained by the infant was apparently adequate since the weight increments were satisfactory and no supplements were necessary.

After the first month in each case supplements of 5 mg. daily crystalline thiamin chloride [2] were ingested by the subjects and a new assay of the milk carried out. A third period of 1 month followed during which still larger supplements of the

TABLE 2

The assay for vitamin B_1 of human milk as affected by thiamin chloride intake

ASSAY	SUBJECT	DAILY SUPPLEMENT OF THIAMIN CHLORIDE	AMOUNT OF MILK FED PER RAT PER DAY	STANDARD GROUP	NUMBER OF RATS	GAIN IN 4 WEEKS	VITAMIN B_1 PER 100 GM.	
		mg.	*ml.*			*gm.*	*μg.*	*I.U.*[1]
1	A	None	7.7		4	28	32	13
Dec. 22, 1937 to				Thiamin chloride, 2.2 μg.	8	25		
Jan. 19, 1938				2.5 μg.	8	28		
				Intern. stand., 10 mg.	9	27		
			8.5	Cow's milk, raw	7	26	27	11
2	A	5	6.0		4	4		
Feb. 5 to Mar. 5, 1938	B	None	10.0		4	13	12	5
				Intern. stand., 10 mg.	6			
			8.4	Cow's milk, pasteurized	4	19	20	9
3	A	10	8.4		7	26	25	11
Feb. 25, to Mar. 25, 1938				Thiamin chloride, 3.0 μg.	5	38		
			8.4	Cow's milk, pasteurized	4	35	32	13
	B	5	8.5		8	22	20	9
				Yeast, 80 mg.	4	52	5000	2160
4								
Apr. 23 to May 21, 1938	B	14.2	8.5		8	19	25	11
				Intern. stand., 10 mg.	4	20		

[1] In assays 1 and 3 both international standard and pure thiamin chloride were used as standards of comparison. In assays 2 and 4, only the international standard was used but the conversion factor was taken as 2.3 instead of 3 because this was the ratio found in the other two assays.

[2] The thiamin chloride under the name of Betaxin was supplied through the generosity of the Medical Research Department of the Winthrop Chemical Company.

thiamin chloride were taken. The details of the biological assays are given in table 2.

It will be noted that the milk produced by subject A during the first period was of high B_1 potency, slightly better than that of the raw cow's milk fed during the same assay. This was evidently a peak period of production for this subject.

Typical menus for 2 days taken by this subject during the first period are given here because of the interest attached to the food intake on which such a high vitamin B_1-containing milk was produced:

FIRST DAY:		SECOND DAY:	
Breakfast	oz.	*Breakfast*	oz.
Grapefruit	2½	Orange juice	4½
Skim milk	9½	Skim milk	7½
Whole milk	2½	Top milk	2½
Wheat germ cereal	3½	Whole wheat bread	1½
Raisin bread	1½	Wheat germ cereal	1½
Sugar	½	Jam	½
Evening lunch	oz.	*Evening lunch*	oz.
Milk	16	Skim milk	9
Egg	2	Cookie	½
Orange	2½	Dates	1
Whole wheat bread	1½	Candy	1
Peanut butter	1		
Sugar	½		
Luncheon	oz.	*Luncheon*	oz.
Cheese	½	Egg	2½
String beans	1½	Orange	2½
Lettuce	1	Skim milk	9
Apple	2½	Cottage cheese	1
Whole milk	9	Wheat bread	1½
Biscuit	1	Apple sauce	2
Cake	1	Peanut butter	½
		Cookie	1
Dinner	oz.	*Dinner*	oz.
Shrimp salad	6	Potato	3½
Cauliflower	2	Beef	3
Baked beans	2	Squash	2
Squash	2	Canned cherries	4
Apple sauce	3	Rutabaga	2
Skim milk	6	Lettuce	1½
Cake	2	Raisin bread	1
		Jam	½
		Mayonnaise	½

In her second period while she was taking 5 mg. thiamin chloride daily, subject A voluntarily reduced her dietary intake of vitamin B_1 since it was assumed that the large supplement would make up for such reduction. But the vitamin B_1 of the milk produced was apparently reduced since daily doses of 6.0 ml. did little more than maintain the weight of depleted rats whereas in the former period 7.7 ml. doses produced as good gains as 10 mg. of the International clay adsorbate.

In the third period with a daily supplement of 10 mg. thiamin chloride subject A produced milk nearly as good as in the first period. For this subject it is obvious that the thiamin supplement offered no advantage. Possibly the maximum concentration of vitamin B_1 had been attained previous to the supplementation.

Subject B on the other hand produced a milk of low vitamin B_1 value during the first period, 11 μg. per 100 gm. This is in the range reported by Neuweiler ('38). Supplements of 5 and then of 14.2 mg. of crystalline thiamin chloride produced increases to 20 and 25 μg. per 100 gm. This subject reported that during the periods of supplementation the constipation from which she and the baby had suffered as a result perhaps of continued use of a non-residue diet, was immediately relieved. The amount of increase in the vitamin B_1 secreted in the milk was sharply limited. If the volume of milk produced daily be taken as 600 gm. in the second period 0.12 mg. of vitamin B_1 was secreted into the milk out of 5 mg. increased intake, and in the third period 0.15 mg. was similarly secreted out of 14.2 mg. increased intake.

The cow's milk values found in these assays, 27, 20 and 32 μg. per 100 gm., are in the range usually reported. No difference between the vitamin B_1 potencies of raw and pasteurized market milk can be deduced from these few observations.

DISCUSSION OF FOOD INTAKE AND MILK PRODUCTION[8]

The typical diet of subject A during the first period contained 1650 μg. of vitamin B_1, yielded 2230 calories and 90 gm.

[8] Our thanks are due to Dr. George R. Cowgill for suggestions on the preparation of this manuscript.

of protein, 60% of which was from milk, egg or meat. There were 90 gm. of fat yielding 810 calories and 265 gm. of carbohydrate yielding 1060 calories. Her estimated calorie requirement not including milk production was 1800 calories. Since her average daily milk production was 840 ml. with probable value of 500 calories the transformation of food into milk calories appears to have been accomplished with but little energy cost.

The total food taken by this subject is small in amount compared with the intakes recorded by Shukers, Macy, Donelson, Nims and Hunscher ('31) for their three subjects, but the latter were producing extraordinary daily amounts of milk, 1420, 2366 and 3134 ml. respectively, on intakes of 3800, 4500 and 4200 calories. The caloric intake and milk production of our subjects represent probably a more nearly normal performance for women supporting only one infant.

The total non-fat calories metabolized by subject A was 1420 at an estimated cost of 426 μg. of vitamin B_1 and 269 μg. of vitamin B_1 appeared in the milk. A positive balance of 955 μg. remains. If however the cost of transference of B_1 to milk be treble that of maintenance as has often been suggested from observation on rats, the margin is reduced to 537 μg., and if the subject's own maintenance requirement be similarly trebled because of lactation demands, the margin is wiped out and a deficit left.

It seems reasonable to assume however that this subject was in vitamin B_1 equilibrium during this period and that her maintenance need for 400 to 500 μg. and her production in milk of 200 to 300 μg. were met by an intake of approximately two and one-half times this amount of the vitamin.

In the third period A produced only 300 calories of milk daily and transferred to it only 125 μg. of vitamin B_1 in spite of the fact that she was receiving 10 mg. of thiamin chloride as well as 1250 μg. in her diet. If her maintenance need was the same as in period I and the same ratio of transformation held, at least 125 μg. of the crystalline synthetic thiamin chloride must have been utilized.

Less exact data as to food intake and milk output are available for subject B. However her diet was considerably richer in carbohydrate than that of subject A, with probably increased maintenance requirement of vitamin B_1. If this be taken as 500 μg., with milk content of 66 μg. her transformation efficiency in period I was better than that of subject A or else she was in negative vitamin balance. By the same type of calculation it might be deduced that in periods 2 and 3 when she received daily 5 and 14.2 mg. thiamin chloride and produced 120 and 150 μg. of vitamin B_1 in her milk little or none of the added thiamin chloride was utilized for the extra milk vitamin. The actual increase in milk vitamin produced was only about 54 and 84 μg. daily.

Since the infants in both cases grew and maintained normal appetites and were at all times in excellent health it is probable that they were receiving sufficient vitamin B_1. They received only the usual supplements of orange juice and cod liver oil, except that in periods 2 and 3 A's baby was given also small amounts of cooked cereal. If total non-fat calories of human milk be estimated at 34 per 100 ml. and if the thiamin (in micrograms) to non-fat calories ratio of Williams and Spies ('38) be used, that is 0.3, no danger of beri beri occurs when the milk carries 10 μg. of thiamin per 100 ml. According to this criterion subject B in period 1 produced a milk barely adequate to prevent beri beri in her infant. The immediate improvement in appetite and intestinal regularity exhibited by this infant during the following periods of supplementation is contributory evidence to the plausibility of this suspicion.

It may be concluded that an intelligent selection of ordinary foods such as that made by A in period I will insure an intake of vitamin B_1 ample for the needs of lactation without fortification or supplementation. Likewise a somewhat restricted diet such as that taken by subject B may bring about low vitamin B_1 content of the breast milk. In the former case large doses of thiamin chloride hampered rather than improved the excretion of B_1 into the milk, and in the latter case the increase in the milk vitamin was not great and probably

no more than could be obtained by increased efficiency in the use of the vitamin of the food. Perhaps the crystalline thiamin chloride when given in such large excess is mostly wasted through non-absorption as found by Schultz, Light and Frey ('38). They were able to detect only about 1 mg. in the urine when 5 mg. thiamin chloride were given daily and about 2 mg. when 10 mg. were given. The rest was found in the feces.

Our subject A appeared however to excrete less vitamin into the milk when these large supplements were given than when natural food mixtures only were used. Perhaps the concentrated thiamin in the intestine had an adverse effect upon absorption of food thiamin.

SUMMARY

Two healthy young women taking diets of widely different vitamin B_1 content were found to secrete milk of similarly different vitamin B_1 potency, 32 and 11 μg. per 100 gm.

When each of these subjects took 5 mg. of crystalline thiamin chloride daily during a period of 1 month no increase occurred in the vitamin B_1 of the milk of the higher potency but some rise was seen in that of lower potency. The new level of the latter milk was 20 μg. per 100 gm.

The first subject then took 10 mg. and the second 14.2 mg. thiamin chloride for a month. The new levels were 25 and 25 μg. per 100 gm.

Three assays of market samples of cow's milk showed 27, 30 and 32 μg. per 100 gm.

It is concluded that the level of vitamin B_1 in human milk is controlled in the lower brackets by the vitamin B_1 content of the diet but that as in cow's milk a maximum level exists above which the vitamin content cannot be raised even by massive doses of thiamin chloride. This maximum level appears to be the same in human and cow's milk, 25 to 32 μg. per 100 gm. of milk.

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THE COMPARATIVE TOXICITY OF FLUORINE IN CALCIUM FLUORIDE AND IN CRYOLITE [1, 2]

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(Received for publication March 29, 1939)

The first toxic effect that appears in animals receiving fluorine compounds in water or food, and the one that persists longest as decreasing doses of fluorine are given, is a hypoplasia of the dental enamel known as 'mottled enamel' in its human manifestation. An experimental and histologic study of this condition in rats by Schour and Smith ('35), revealed that fluorine has a direct disturbing effect on the enamel epithelium and later a more severe effect on the formation of the organic matrix. Neither of these effects is the same as those of other dental disturbances of nutritional (vitamin A deficiency) or of endocrine (parathyroidectomy, hypoparathyroidism, hypophysectomy) origin.

The concentrations of fluorine in municipal water supplies that constitute public health hazards, solely with reference to the incidence of mottled enamel however, have been well defined. For public health purposes, Dean and Elvove ('35) have defined the minimal threshold of fluoride concentration in a domestic water supply as "the highest concentration of fluoride incapable of producing a definite degree of mottled enamel in as much as 10% of the group examined." They

[1] This experiment was made possible by the donation of funds to the University of Illinois by the Aluminum Company of America.

[2] This investigation was conducted under the supervision of a Committee on the Physiological Effects of Spray Chemicals, appointed by the director of the Agricultural Experiment Station and consisting of the following members: H. H. Mitchell, W. A. Ruth, W. P. Flint and Julia P. Outhouse.

present evidence indicating that this critical concentration, determined on samples collected over a 12-month period, is approximately 1 part per million or slightly less, and this seems to be the concensus of competent opinion. Thus, the toxicity of fluorine in drinking water has been quite definitely determined, insofar as toxicity can be expressed in terms of concentration. While it must be true that the amount of fluorine-bearing water consumed is an important factor in the production of toxic effects, perhaps the all-important factor, the public health evaluation of a specific water supply must of course be based upon its content of potentially dangerous elements.

The use of fluorine compounds, such as cryolite (Na_3AlF_6) and barium fluosilicate in spraying fruits and vegetables for the purpose of insect control, represents another type of health hazard insofar as these compounds remain on the surface of the marketed food and are consumed. In recognition of this fact, the secretary of agriculture has imposed a legal tolerance on such spray residues of 0.02 grain of fluorine per pound of food, equivalent to about 2.9 p.p.m. This tolerance applies only in interstate commerce.

Since the toxicity of fluorine in water has been so clearly determined, within the limits of accuracy imposed by the only practical method of expression, it would seem feasible to estimate the toxicity of fluorine spray residues by determining, first, the relative toxicity of the fluorine compounds occurring in water and those used in fruit and vegetable sprays, and second, the relative toxicity of fluorine in the same chemical combination but consumed in water and in food. The present paper will be concerned with the first of these problems as it relates to cryolite, while the following paper will be concerned with the second.

In comparing the toxicity of fluorine as cryolite with that of fluorine in potable waters, it was assumed that the latter, insofar as it is in a non-ionized condition, is in the form of calcium fluoride, because 1) many of the naturally-occurring fluoride-bearing rocks, which would be the ultimate source

of the water-borne fluorine, contain their fluorine in combination with calcium, 2) calcium fluoride is the least soluble fluoride commonly found in nature, and 3) the solubility of calcium fluoride would permit concentrations of the element at 18°C. of about 8 p.p.m., and very few potable waters in this country contain more fluorine than this. Cryolite is many times more soluble than calcium fluoride, the relationship being 15 to 1 according to Carter ('28) and 23 to 1 according to Roholm ('37).

The solubility of inorganic salts does not necessarily determine their absorbability from the mammalian intestinal tract, except for compounds so slightly soluble that they are suitable for gravimetric chemical analysis. Furthermore, the differences in absorbability that are dependent upon differences in solubility would be expected to diminish as the amounts of the compounds consumed diminish. Confirming this expectation, De Eds and Thomas ('34) found that wide differences in the solubility of fluorine compounds did not occasion appreciable differences in the minimum toxic concentrations for rats. Smith and Leverton ('34) have come to essentially the same conclusion, namely, that at a concentration of 14 p.p.m., fluorine, regardless of the solubility of the compound used, gives the first outwardly discernible effect upon the incisor teeth of the rat that was regularly observed. However, there were indications in the latter experiment that, even at this low concentration, fluorine was more toxic in the form of sodium fluoride and barium fluosilicate than in the form of calcium fluoride and cryolite.

Obviously only these low levels of fluorine intake are of significance to the problem of the toxicity of fluorine in drinking water and in spray residues. Furthermore, since only small differences in toxicity between different fluorine compounds are to be expected at low levels of feeding, the compounds to be compared should be fed in water solution to avoid complications due to particle size. Also the consumption of both food and fluorine-bearing water should be equalized between comparative animals so that equal dosages of fluorine

will be consumed as well as equal amounts of food, since it is conceivable that a variable intake of food would modify the physiological effect of a given dose of fluorine. The experiment to be reported in this paper was planned according to these conceptions. Young albino rats served as subjects of the experiment, with the assurance that the data obtained as far as the comparative toxicities of CaF_2 and Na_3AlF_6 are concerned, are applicable to humans, since dental fluorosis in the rat and in the human are quite similar (Schour and Smith, '35).

PLAN OF THE EXPERIMENT

Twenty-four albino rats, averaging initially 57 gm. in weight, were divided into twelve pairs on the basis of sex, body weight and litter membership. They were fed individually by the paired-feeding technic on a ration of Purina Fox chow of the following percentage composition: dry substance 90.98, ether extract 5.68, crude fiber 3.48, nitrogen 3.96, ash 7.65, calcium 1.24 and phosphorus 0.86. The heat of combustion was 4.166 calories per gram and the fluorine content 3.04 p.p.m.

The cryolite used in this experiment was a synthetic product marketed as an insecticide. It contained 43.7% fluorine and about 15% of non-cryolite material, including hydrated oxide of aluminum and compounds of sodium, calcium and silicon. The calcium fluoride was a chemically pure product.

Each pair of rats received the same amount of food daily and the same amount of fluorine in the drinking water. The odd-numbered rats received water containing CaF_2 and the even-numbered rats water containing cryolite. In order to avoid any possibility of the solid food depressing or obscuring the toxic effects of the test substances administered in the water, the consumption of the food and of the water was separated as much in time as possible. The rats were allowed to eat from 8 to 9.30 A.M. and during this time the water consumption was restricted to the 4 to 5 cc. of distilled water that was mixed with the food to prevent scattering. From

9.30 A.M. to 6 P.M. the rats had access to neither food or water. At 6 P.M. they were given measured portions of the fluorine-containing water and after these were consumed they were given distilled water ad libitum until the following morning at 8 A.M.

To permit an estimate of the initial fluorine content of the experimental rats, six rats of approximately the same weight range as the experimental rats were analyzed after removal of the contents of the alimentary canal. The entire carcasses of these rats were analyzed for fluorine by the technic used with the soft-tissue samples of the experimental rats.

After approximately 14 weeks of feeding, the rats were killed with ether and the digestive tracts removed, emptied, washed with distilled water and replaced. After determining the empty weights, the carcasses were autoclaved at 12 pounds steam pressure for 30 to 45 minutes in order to facilitate the separation of the skeleton from the soft tissues. The latter were combined with all condensed water, finely ground and thoroughly mixed by hand. The bones, after removal of the teeth, were dried for 12 hours at 110°C., broken up and extracted with ether for 48 hours in a Soxhlet extractor. The extracted bone was then dried at 110°C. for 20 minutes, weighed and finely ground in a hand mill. The teeth, after removal from the carcass, were washed first in water to remove all adhering tissue, and then in ether, after which they were dried at 110° for 24 hours and weighed.

The soft tissue, bones and teeth were analyzed separately for fluorine. Samples of soft tissue were weighed out, mixed with 15 cc. of a 10% solution of magnesium acetate and ashed for 12 to 16 hours at temperatures of 500° to 550°C. in an electric muffle furnace. Samples of bone were ashed for 16 to 20 hours at 550°C., while the entire weight of teeth was ashed for 20 hours at 550° to 600°C., carefully ground in an agate mortar and re-ashed for 24 hours at the same temperature.

The fluorine was isolated from the ash of all samples by distillation as hydrofluosilicic acid according to the well-known

method of Willard and Winter ('33), and a duplicate distillation was carried out to remove completely all phosphates, in accordance with the recommendations of Churchill, Bridges and Rowley ('37). The first distillation was made with sulfuric acid and the second with perchloric acid. The distillation temperature was maintained at 140°C. by the introduction of steam at the proper rate into the distillation flask. The distillation was continued until 250 cc. of liquid had been collected. In the analysis of soft-tissue ash the chloride ion was removed from the first distillate by precipitation with $AgClo_4$, as suggested by Armstrong ('36).

The second distillates were made alkaline with a dilute solution of NaOH and were then evaporated on an electric hot plate at a temperature just below boiling. In the case of the bone samples, the Rowley and Churchill ('37) procedure was followed, employing an aqueous medium for the titration of the fluorine with thorium nitrate. In the case of the soft tissue and tooth samples, the micro method of Armstrong ('36) was used, which permits the determination of as little as 10 micrograms of fluorine in 10 cc. of distillate. The only departure from Armstrong's directions was the use of a stronger thorium nitrate solution, 0.0006 to 0.0007 N, instead of 0.0004 N.

A number of recovery tests were undertaken with the analytical procedures above described. In five tests with bone samples, to which fluorine was added in amounts equal to those expected in the samples themselves, the recoveries ranged from 96 to 101%, averaging 98.5. In five tests with soft-tissue samples conducted on the same principle, the percentage recoveries ranged from 92 to 100, and averaged 95.4.

RESULTS OF THE EXPERIMENT

Because of the imposition of the above-described severe experimental conditions, the food consumption of the rats was low and the daily gains in body weight averaged only about 0.9 gm. However, the rats showed no other adverse effects except during the first week of the experiment. At this time

several of the rats, irrespective of the nature of the fluorine supplement received, showed a hematuria lasting 1 or 2 days.

The teeth of the rats were examined once a week during the period of experimental feeding, using a jeweler's lens with a magnification of 4x. Striations of the enamel began to appear during the eighth week of feeding and were visible on the teeth of all rats by the end of the tenth week of feeding. In all probability tooth striations would have appeared earlier if the rats had consumed the experimental diets better. No distinction could be made between the rats receiving CaF_2 and those receiving Na_3AlF_6 with reference to tooth appearance.

In table 1 will be found a summary of the growth data and of the data on fluorine retention. The growth rates of the rats were not affected to an appreciable extent by the nature of the fluorine supplement fed, nor were the appetites of the rat for food, as judged by the numbers of food refusals (not given in the table). The weight of dry, fat-free bone and of dried teeth were also not affected by the difference in fluorine supplement given the paired rats.

The fluorine intake of the rats was equivalent on the average to 13.0 parts per million of solid food consumed, or 0.75 mg. per kilogram of body weight daily. Seventy-seven per cent of the fluorine consumed was contained in the drinking water and 23% in the basal diet.

The concentration of fluorine in the bones averaged 620 parts per million of dry, fat-free substance, and that in the teeth 245 p.p.m. of dry substance. The observed concentration of fluorine in the soft tissues was not significant because of the variable amounts of condensed water in these samples. The weights of the samples may be estimated, however, from the empty weight of carcass, the weight of dry fat-free bones and the dry weight of teeth. From another series of twelve rats, averaging 220 gm. in weight, the total dried, fat-free skeleton averaged 50.6% of the fresh weight. The teeth were assumed to contain 14% of moisture (Matsuda, '27). Using these factors and the average fluorine content

TABLE 1

The results on growth and fluorine retention

Pair number and sex	1♀		2♀		3♀		4♂		5♂		6♀	
Rat number	1	2	3	4	5	6	7	8	9	10	11	12
Fluorine compound fed	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite
Period of feeding, days	97	97	98	98	97	97	97	97	99	99	100	100
Total food consumed, gm.	636	636	768	768	599	599	665	665	502	502	646	646
Total gain in body weight, gm.	61	80	68	72	68	69	110	111	75	80	82	77
Final empty body weight, gm.	134	152	113	119	125	129	162	163	122	127	135	131
Total weight of bones,[1] gm.	7.522	7.367	7.216	7.261	6.554	7.489	7.995	7.435	6.676	6.458	7.930	6.755
Total weight of teeth,[2] gm.	0.399	0.400	0.374	0.382	0.366	0.367	0.356	0.363	0.344	0.354	0.367	0.362
Fluorine in bones, p.p.m.	649	645	634	630	646	590	743	795	573	582	570	700
Fluorine in teeth, p.p.m.	294	261	233	255	250	221	295	255	215	196	188	249
Fluorine ingested in food, mg.	1.909	1.909	2.304	2.304	1.797	1.797	1.997	1.997	1.508	1.508	1.938	1.938
Fluorine ingested in water, mg.	7.153	7.154	5.446	5.447	5.691	5.690	7.877	7.877	4.475	4.475	6.044	6.044
Total fluorine ingested, mg.	9.062	9.063	7.750	7.751	7.488	7.487	9.874	9.874	5.983	5.983	7.982	7.982
Fluorine in bones, mg.	4.882	4.752	4.575	4.574	4.234	4.419	5.940	5.911	3.825	3.759	4.520	4.728
Fluorine in teeth, mg.	0.117	0.104	0.087	0.097	0.092	0.081	0.105	0.092	0.074	0.067	0.069	0.090
Fluorine in soft tissues, mg.	0.127	0.132	0.083	0.071	0.077	0.065	0.136	0.126	(0.081)	(0.475)	0.111	0.098
Total fluorine in carcass, mg.	5.128	4.988	4.745	4.742	4.403	4.565	6.181	6.129	3.980	4.304	4.700	4.916
Estimated fluorine retention,[3] mg.	5.009	4.873	4.673	4.667	4.312	4.469	6.098	6.046	3.905	4.229	4.615	4.830
Per cent retention of fluorine	55.3	53.8	60.3	60.2	57.6	59.7	61.8	61.2	65.3	70.7	57.8	60.5
Per cent of retained fluorine in bones	95.2	95.3	96.4	96.4	96.3	96.8	96.1	96.4	96.1	...	96.2	96.2

TABLE 1—*Continued*

Pair number and sex	7 ♀		8 ♂		9 ♂		10 ♂		11 ♂		12 ♂		Averages	
Rat number	13	14	15	16	17	18	19	20	25	26	27	28	Odd	Even
Fluorine compound fed	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite	CaF_2	Cryolite
Period of feeding, days	103	103	105	105	100	100	100	100	96	96	95	95	99	99
Total food consumed, gm.	756	756	652	652	642	642	684	684	537	537	628	628	643	643
Total gain in body weight, gm.	83	75	79	63	125	120	127	106	112	126	99	95	91	90
Final empty body weight, gm.	158	151	152	138	175	171	173	153	162	176	154	152	147	147
Total weight of bones,[1] gm.	8.703	8.180	8.183	8.746	9.172	7.562	8.570	7.976	8.798	8.511	7.147	8.368	7.872	7.676
Total weight of teeth,[2] gm.	0.370	0.434	0.433	0.392	0.382	0.395	0.382	0.364	0.371	0.352	0.354	0.358	0.375	0.377
Fluorine in bones, p.p.m.	627	630	569	575	566	635	655	683	527	540	613	502	614	626
Fluorine in teeth, p.p.m.	306	238	195	269	276	250	271	248	201	209	278	223	250	240
Fluorine ingested in food, mg.	2.270	2.270	1.958	1.958	1.928	1.928	2.054	2.054	1.611	1.611	1.884	1.884	1.930	1.930
Fluorine ingested in water, mg.	8.142	8.142	6.716	6.717	6.554	6.554	7.174	7.174	5.866	5.866	6.118	6.117	6.438	6.438
Total fluorine ingested, mg.	10.412	10.412	8.674	8.675	8.482	8.482	9.228	9.228	7.477	7.477	8.002	8.001	8.368	8.368
Fluorine in bones, mg.	5.457	5.153	4.654	5.029	5.191	4.802	5.613	5.448	4.637	4.596	4.381	4.201	4.826	4.781
Fluorine in teeth, mg.	0.113	0.103	0.084	0.105	0.105	0.099	0.104	0.090	0.075	0.074	0.098	0.080	0.094	0.090
Fluorine in soft tissues, mg.	0.103	0.110	0.093	0.100	0.159	0.131	0.111	0.080	0.121	0.095	0.100	0.089	0.111	0.100
Total fluorine in carcass, mg.	5.674	5.366	4.831	5.234	5.455	5.032	5.828	5.619	4.833	4.765	4.579	4.370	5.028	5.002
Estimated fluorine retention,[3] mg.	5.554	5.245	4.712	5.114	5.375	4.951	5.754	5.544	4.753	4.685	4.491	4.279	4.938	4.911
Per cent retention of fluorine	53.3	50.4	54.3	59.0	63.4	58.4	62.4	60.1	63.6	62.7	56.1	53.5	59.3	59.2
Per cent of retained fluorine in bones	96.2	96.0	96.3	96.1	95.2	95.4	96.3	97.0	96.0	96.5	95.7	96.1	96.0	96.2

[1] Dry, fat-free weight.
[2] Weight after drying at 110°C.
[3] The initial fluorine content of the rats was estimated at 1.6 microgram per gram.

of the soft tissues (0.105 mg.), it may be shown that the concentration of fluorine in the soft tissues averaged 0.71 p.p.m.

The nature of the fluorine supplement had no appreciable effect upon these concentrations of fluorine in the tissues. The averages for the cryolite rats and the calcium fluoride rats were not greatly different and among the twelve pairs of rats there was no consistency in this respect. The pairs divided eight to four in favor either of the cryolite (for bone) or of calcium fluoride (for teeth and soft tissues). In such a situation the application of statistical analysis is unnecessary.

The total quantities of fluorine recovered in the carcasses of the experimental rats were quite similar for the rats on the two fluorine supplements, averaging 5.028 mg. for the rats receiving calcium fluoride and 5.002 mg. for the rats receiving cryolite. The amounts of fluorine retained during the experimental feeding period were estimated by deducting from the amounts of fluorine found, the amounts estimated to have been present in the rats at the start of the feeding period. The six check rats killed at this time contained the following amounts of fluorine in micrograms per gram of weight: 2.4, 1.9, 1.5, 1.5, 1.3 and 1.1, averaging 1.6. The initial content of fluorine was estimated at 1.6 micrograms per gram initial weight.

The quantities of fluorine retained during the experimental feeding period averaged 59.3% of the amount fed for the rats receiving the calcium fluoride supplement, and 59.2% for the rats receiving the cryolite. Among the twelve pairs, four showed the greater percentage retention for the cryolite rats, and with three others the difference was less than 1%. The data, therefore, do not reveal any appreciable difference between the rate of retention of fluorine from calcium fluoride and from cryolite.

Over 96% of the fluorine retained by these rats was deposited in the skeleton, and this quota was remarkably constant among the twenty-four rats, the percentage varying

only from 95.2 to 97.0. The remainder of the recovered fluorine was deposited in about equal amounts in the teeth and in the soft tissues, 1.85 and 2.05%, respectively.

CONCLUSIONS

The fluorine in cryolite is no more toxic to growing rats nor is it retained in the body to a greater extent, than the fluorine in calcium fluoride when both are administered in aqueous solution at the rate of 0.58 mg. per kilogram of body weight daily. The appearance of striations in the incisor teeth is equally rapid with both fluorine compounds.

About 96% of the fluorine retained at an intake equivalent to 13 p.p.m. of food consumed is deposited in the skeleton, and the remaining 4% is about equally divided between teeth and soft tissues.

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A COMPARISON OF THE TOXICITY OF FLUORINE IN THE FORM OF CRYOLITE ADMINISTERED IN WATER AND IN FOOD[1,2]

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In the preceding paper it was shown that the fluorine in cryolite is not appreciably different in toxicity for growing rats from that in calcium fluoride (the form in which fluorine probably occurs in most fluorine-bearing waters insofar as it is present in combination), when both compounds are administered in the drinking water at a low level, 0.58 mg. per kilogram daily. The next step in determining whether the fluorine in cryolite (Na_3AlF_6) in the form of spray residue on fruits and vegetables is any more or less toxic than the fluorine in drinking water, the relation of which to malformation of dental enamel has been quantitatively assessed, is a comparison of the toxicity of the same fluorine compound when administered in water and in food.

Dean, Sebrell, Breaux and Elvove ('34) observed that 500 p.p.m. of NaF in the drinking water exhibited in rats a relatively greater toxicity, as evidenced by growth rate and tooth structure, than the same concentration of fluorine in the diet under their experimental conditions. They state further: "Whether this result is due to differences in the total quantity of sodium fluoride ingested, to more complete absorption,

[1] This experiment was made possible by the donation of funds to the University of Illinois by the Aluminum Company of America.

[2] This investigation was conducted under the supervision of a Committee on the Physiological Effects of Spray Chemicals, appointed by the director of the Agricultural Experiment Station and consisting of the following members: H. H. Mitchell, W. A. Ruth, W. P. Flint and Julia P. Outhouse.

to a more rapid rate of absorption or to other factors has not been definitely determined." Marcovitch, Shuey and Stanley ('37) concluded that sodium fluoride affects the teeth of rats at a lower concentration in the drinking water (4 p.p.m.) than in the food (7 p.p.m.), and at a still lower concentration in the drinking water (1 p.p.m.) when the food is cooked to dryness in 3.4 times its weight of such water. In the following year Marcovitch and Stanley ('38) reported that rats receiving 4 p.p.m. of fluorine in the drinking water as sodium fluoride retain nearly twice as much of the element as rats receiving 4 p.p.m. of fluorine as cryolite in the diet, admitting that: "This is due in part to the greater consumption of fluorine in the drinking water, but no doubt solubility is also a factor."

Experiments in which the comparison of the toxicity of fluorine in drinking water and in food is made with animals receiving unrestricted amounts of both, cannot be expected to answer the question whether the method of administration of fluorine per se is a factor. Nor can the results of such experiments be presumed to possess any wide range of applicability in practical human nutrition, in which water and food are both consumed ad libitum. The ratio of the voluntary consumption of water to that of food is subject to such wide variation on account of differences in the character of the food, the environmental temperature, the activity of the subject and possibly the species of animal as well as other factors, that experimental results pertaining to any particular ratio secured under one set of conditions cannot be applied to other ratios and other conditions.

The experiments to be described below were planned to answer the question whether the method of administration of fluorine per se, in water or in food, modified its toxicity as measured by changes in the dental enamel and by its retention in the body. The source of fluorine in both cases was cryolite.

PLAN OF EXPERIMENT

Twelve pairs of young albino rats ranging in weight from 60 to 90 gm. were chosen from litter mates of the same sex

and approximately the same weight. They were fed according to the paired-feeding technic upon a basal diet, consisting of dried beef powder 25%, dried yeast 8%, sucrose 10%, lard 8%, butterfat 7%, cod liver oil 1%, a calcium-free salt mixture[3] 3%, $CaCO_3$ 1%, and starch 37%. This diet contained 0.88 p.p.m. of fluorine.

The even numbered rats in each pair received this ration with enough cryolite added to bring the fluorine concentration to 10 p.p.m. This required 9.1 mg. of F per kilogram of diet. A solution containing 9.1 mg. of F, as cryolite, per liter was made up to supply the fluorine supplement to the odd numbered rats in each pair. Each odd numbered rat received daily as many cubic centimeters of this solution as it received in grams of the basal diet. Hence, the rats in each pair received the same amount of food daily and the same dosage of fluorine as cryolite, one in solid form mixed into its food and the other in aqueous solution. The cryolite used was a synthetic product that is being marketed as an insecticide. It contained 43.7% of fluorine. As originally obtained, it all passed through a 200 mesh sieve, and during the process of mixing with the starch ingredient of the diet, it was further pulverized in a ball mill for 3 or 4 hours.

In order to obtain the maximum effect of the difference in the method of administration of the fluorine, it seemed essential to separate as far as possible the times of consumption of food and of cryolite solution. Hence, the following schedule was followed. The rats were fed at 4 o'clock in the afternoon and were allowed to eat until 7 o'clock. In order to prevent scattering, each day's portion of food was moistened with about 4 cc. of distilled water, but otherwise the rats received no water until 8 o'clock in the morning. The even-numbered rats received redistilled water ad libitum from 8 A.M. to 4 P.M. The odd-numbered rats received their daily quota of cryolite solution at 8 A.M. and, when this was consumed, as much redistilled water as they cared for until 4 P.M.

[3] Containing no fluorine and, besides the ordinary ingredients, cobalt chloride, zinc chloride and copper sulfate.

Fluorine balance experiments were run on all pairs of rats at least once, and twice for four pairs, in the interval from the tenth to the nineteenth weeks of the experiment. Each collection period lasted 14 days, during which time the rat was confined in a Pyrex crystallizing dish, measuring 190 mm. in diameter and 100 mm. in height. The bottom of the collection dish was covered with a circular filter paper for the absorption of urine, over which was placed a fine metal screen for the collection of the feces. The rat was supported on a metal grid with ½ inch mesh, raised about 2 inches above the bottom of the dish by metal supports. The dish was covered by an inverted cylindrical cage made of hardware cloth and standing about 7.5 inches high. The papers for the absorption of urine were removed every other day, moistened with magnesium acetate solution and dried. The dishes and grids were washed with hot redistilled water several times during each collection period. The washings were made alkaline with calcium hydroxide and evaporated to dryness.

At the termination of the collection period, the treated filter papers were placed within the proper beakers containing the residue from the urine washings, and the papers and residue were ashed at 500°C. in an electric muffle furnace. The feces collections were treated with a solution of magnesium acetate, dried and ashed at the same temperature. Fluorine determinations on feces and urine were made according to the micro-method of Armstrong ('36).

In some of the balance experiments, the urine collections were divided into two periods, from 8 A.M. to 5 P.M. and 5 P.M. to 8 A.M., in order to detect differences in the rate of urinary excretion between the rats receiving their fluorine supplement at 4 P.M. (in the food) and those receiving their supplement at 8 A.M. (in the water).

The rats were killed with ether at the end of 19 or 20 weeks of experimental feeding. The empty weight was determined and the carcasses separated into soft tissues, skeleton and teeth as described in the preceding paper. The fluorine was

determined in each of these samples according to the methods previously given.

EXPERIMENTAL RESULTS

The physical condition of the rats, the rate of growth and the appearance of the teeth were observed throughout the feeding period. The growth of the rats was uniformly slow because of the adverse feeding conditions that were of necessity imposed. The average daily gain in body weight was only 0.83 gm. Food refusals were frequent and in eleven of the twelve pairs were more frequent for the rat receiving its cryolite in the water, indicating a definite impairment of appetite by this method of fluorine administration. In the average, the food refusals were more than twice as frequent for the rats receiving the cryolite solution than for those with cryolite in their diet.

Striations appeared on the lower incisor teeth starting with the seventh week of feeding and by the tenth week all of the rats showed this effect. In four pairs of rats, the rat receiving cryolite in water showed tooth striations first, in four pairs the reverse was true, and in four pairs striations were observed at the same time in both rats. Thus, as far as these weekly examinations of the teeth revealed, there was no difference in the time of appearance of tooth striations between the two methods of fluorine administration.

During the first week or so of the experiment about half of the rats receiving fluorine in their drinking water exhibited hematuria that recurred every few days and then disappeared completely. This phenomenon was observed also in the experiments described in the preceding paper, in all of which fluorine was administered in the water. It evidently represents only a temporary effect upon kidney function, albeit an intense effect while it lasts.

The growth data and the results of the fluorine analyses of the tissues are summarized in table 1. Neither the total gains in body weight made by pair mates, nor the attained body lengths (not summarized in the table), indicate any appre-

TABLE 1

Growth data and fluorine analyses of tissues

PAIR NUMBER AND SEX	1♀		2♀		3♀		4♀		5♀		6♀	
RAT NUMBER	108	109	110	111	112	113	114	115	116	117	118	119
CRYOLITE ADMINISTRATION	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water
Feeding period in days	136	136	136	136	141	141	136	136	136	136	142	142
Total food consumed, gm.	905	905	937	937	971	971	816	816	893	893	816	816
Total gain in body weight, gm.	100	99	97	111	123	126	118	92	102	87	79	89
Final empty body weight, gm.	186	184	176	193	198	199	178	155	191	174	153	165
Total weight of bones,[1] gm.	8.830	10.393	9.692	9.252	8.945	8.955	9.113	8.451	9.867	9.762	9.115	8.479
Total weight of teeth,[2] gm.	0.411	0.437	0.408	0.429	0.440	0.466	0.443	0.403	0.427	0.416	0.401	0.402
Fluorine in bones, p.p.m.	397	464	375	463	392	492	376	498	380	426	368	489
Fuorine in teeth, p.p.m.	208	221	235	247	219	225	190	247	227	260	196	235
Total fluorine ingested, mg.	8.82	8.82	9.26	9.26	9.15	9.15	8.03	8.03	8.72	8.72	7.98	7.98
Fluorine in bones, mg.	3.506	4.823	3.635	4.284	3.506	4.406	3.427	4.209	3.750	4.159	3.355	4.146
Fluorine in teeth, mg.	0.085	0.097	0.096	0.106	0.096	0.105	0.084	0.100	0.097	0.108	0.079	0.094
Fluorine in soft tissues, mg.	0.116	0.160	0.094	0.109	0.120	0.129	0.083	0.089	(0.214)	(0.099)	0.057	0.090
Total fluorine in carcass, mg.	3.707	5.080	3.825	4.499	3.722	4.640	3.594	4.398	4.061	4.364	3.491	4.330
Estimated fluorine retention,[3] mg.	3.569	4.944	3.699	4.368	3.602	4.523	3.498	4.297	3.919	4.225	3.373	4.208
Per cent retention of fluorine	40.5	56.1	39.9	47.2	39.4	49.4	43.6	53.5	44.9	48.5	42.3	52.7
Per cent of retained fluorine in bones	94.6	94.9	95.0	95.2	94.2	95.0	95.4	95.7	92.3	95.3	96.1	95.8

TABLE 1—*Continued*

PAIR NUMBER AND SEX	7♀		8♀		9♀		10♀		11♂		12♂		AVERAGES	
RAT NUMBER	120	121	122	123	124	125	126	127	128	129	130	131		
CRYOLITE ADMINISTRATION	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water
Feeding period in days	142	142	140	140	140	140	135	135	133	133	132	132	137	137
Total food consumed, gm.	853	853	813	813	867	867	900	900	1072	1072	1093	1093	912	912
Total gain in body weight, gm.	81	88	87	89	92	86	100	89	185	181	203	201	114	112
Final empty body weight, gm.	163	168	157	160	154	150	173	160	252	251	279	274	185	186
Total weight of bones,[1] gm.	8.645	9.261	8.752	8.295	10.031	7.721	9.123	8.457	10.541	11.070	12.595	13.165	9.604	9.438
Total weight of teeth,[2] gm.	0.410	0.445	0.437	0.368	0.397	0.397	0.453	0.365	0.431	0.440	0.470	0.418	0.427	0.415
Fluorine in bones, p.p.m.	348	447	347	447	354	472	376	434	336	432	283	365	361	452
Fuorine in teeth, p.p.m.	198	212	179	218	209	212	192	256	227	243	219	251	208	236
Total fluorine ingested, mg.	8.33	8.33	7.92	7.92	8.08	8.08	8.64	8.64	10.10	10.10	10.24	10.24	8.773	8.773
Fluorine in bones, mg.	3.008	4.140	3.037	3.708	3.551	3.644	3.430	3.670	3.542	4.782	3.564	4.805	3.443	4.231
Fluorine in teeth, mg.	0.081	0.094	0.078	0.080	0.083	0.084	0.087	0.093	0.098	0.107	0.103	0.105	0.089	0.098
Fluorine in soft tissues, mg.	0.067	0.082	0.051	0.058	0.068	0.080	0.079	0.083	0.128	0.153	0.153	0.185	0.092	0.107
Total fluorine in carcass, mg.	3.156	4.316	3.186	3.846	3.702	3.808	3.596	3.846	3.768	5.042	3.820	5.095	3.636	4.439
Estimated fluorine retention,[3] mg.	3.025	4.188	3.074	3.732	3.603	3.706	3.479	3.732	3.661	4.930	3.708	4.978	3.517	4.319
Per cent retention of fluorine	36.3	50.3	38.8	47.1	44.6	45.9	40.3	43.2	36.3	48.8	36.2	48.6	40.3	49.3
Per cent of retained fluorine in bones	95.3	95.9	95.3	96.4	95.9	95.7	95.4	95.4	94.0	94.8	93.3	94.3	94.7	95.4

[1] Weight of dry, fat-free bone.

[2] Weight of dry teeth.

[3] Total fluorine in carcass minus estimate of initial content of fluorine, taken as 1.6 p.p.m. of initial weight of body.

ciable effect of the method of fluorine administration upon body growth. Also, no consistent differences in the weight of dry, fat-free bone or of the teeth were obtained between pair mates.

However, the fluorine analyses revealed a greater concentration of fluorine in bone and in teeth in all twelve pairs for the rat receiving its cryolite in water. For twelve events, each of which may result with equal probability in either of two ways, a consistent outcome would be obtained by chance on an average of only twice in 4096 trials (from the binomial distribution), or once in 2048 trials. This probability is so small that it may be neglected, and the consistent outcomes observed can reasonably be ascribed to the difference in experimental treatment deliberately imposed, i.e., the method of fluorine administration.

The contents of fluorine in bone and teeth were also consistently greater for the rat in each pair receiving its cryolite via the drinking water, and the same was true with one exception for the fluorine contained in the soft tissues. The one exception occurs in pair no. 5, in which the soft-tissue sample for the rat receiving cryolite in its food exhibited a fluorine content three or four times as large as any other similar sample. It is very probable that this sample was inadvertently contaminated with a small piece of bone, especially since the observed percentage of retained fluorine deposited in the skeleton was much lower for this rat than for any other. It may be concluded, therefore, that equal doses of fluorine cause a greater deposit of fluorine in bone, teeth and soft tissue when administered in the water than when administered in the food.

The estimates of the amounts of fluorine retained by the rats during the experimental feeding period, using the procedure explained in the preceding paper, were less in all pairs for the rat receiving fluorine in its food, the averages being 3.517 and 4.319 mg., amounting to 40.3 and 49.3%, respectively, of the fluorine consumed. Thus, the retention of fluorine was 18.6% less for the rats receiving their fluorine in

the food than for those receiving it in the water. Since the basal diet supplied 9% of the fluorine intake, the observed average difference in fluorine retention must have been produced, outside the limits of random error, by 91% of the fluorine intake of the odd numbered rats. Hence, if these rats had received all of their fluorine in the drinking water, it may be predicted, neglecting random errors, that instead of retaining 0.802 mg. more of fluorine than their pair mates, they would have stored $0.802 \div 0.91 = 0.881$ mg. more, a difference of 20.4, instead of 18.6%. It may be concluded, therefore, that under the conditions of this experiment the administration of fluorine in food, rather than in water, decreases the retention of fluorine, and hence its potential toxicity, by 20.4%.

It is an interesting fact that in nine of the twelve pairs of rats, the rat receiving its fluorine in the water retained a greater percentage of the stored fluorine in its skeleton than its pair mate, the averages being 95.4 and 94.7, respectively. In one pair, the percentages were the same. In making a statistical analysis of this situation, it seemed fair to exclude the results of pair 5 in which a questionably low percentage was obtained, as explained above, for the rat getting its fluorine in the food. For the remaining eleven pairs, the mean difference in the percentage of fluorine stored that was deposited in the bones was 0.418, favoring the rats receiving the cryolite solution, the standard deviation of differences was 0.495, the value of t, 2.656, and the probability, P, that such an average difference was produced by fortuitous factors only, 0.012 (Student, '25). This probability is so small that it may be neglected. We may conclude, therefore, that the greater storage of fluorine in the body associated with its administration in the drinking water occasioned a greater proportional storage in the skeleton.

The results of the fluorine balance trials, summarized in table 2, substantiate the results of the carcass analyses. In thirteen of the sixteen trials, more of the ingested fluorine

TABLE 2

Fluorine balance data. Results expressed on the 14-day basis

PAIR NUMBER AND SEX	1♀		2♀		3♀		4♀		5♀		6♀	
RAT NUMBER	108	109	110	111	112	113	114	115	116	117	118	119
CRYOLITE ADMINISTRATION	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water
Fluorine balance: Wk. 10 and 11												
F ingested, micrograms	915	915	855	855	885	885	820	820				
F in feces, micrograms	228	92	160	95	252	177	109	171				
F in urine, micrograms	270	277	265	270	260	286	244	244				
Per cent ingested F excreted	54	40	50	43	58	52	43	51				
Fluorine balance: Wk. 13 and 14												
F ingested, micrograms									940	940	805	805
F in feces, micrograms									280	158	220	162
F in urine, micrograms									395	488	277	280
Per cent ingested F excreted									72	69	62	55
Fluorine balance: Wk. 18 and 19												
F ingested, micrograms	885	885	980	980	1035	1035	910	910				
F in feces, micrograms	293	224	327	258	388	293	247	184				
F in urine, micrograms												
8 A.M. to 5 P.M.	26	252	148	285	122	280	27	268				
5 P.M. to 8 A.M.	382	95	309	142	352	197	263	110				
Per cent ingested F excreted	79	64	80	70	83	74	59	62				

PAIR NUMBER AND SEX	7♀		8♀		9♀		10♀		11♂		12♂	
RAT NUMBER	120	121	122	123	124	125	126	127	128	129	130	131
CRYOLITE ADMINISTRATION	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water	In food	In water
Fluorine balance: Wk. 13 and 14												
F ingested, micrograms	770	770	835	835								
F in feces, micrograms	222	149	228	146								
F in urine, micrograms	330	330	432	429								
Per cent ingested F excreted	72	62	79	69								
Fluorine balance: Wk. 14 and 15												
F ingested, micrograms					755	755	915	915	1180	1180	1160	1160
F in feces, micrograms					208	129	250	171	356	223	339	209
F in urine, micrograms												
8 A.M. to 5 P.M.					53	152	27	191	99	317	139	317
5 P.M. to 8 A.M.					218	99	211	135	327	165	271	139
Per cent ingested F excreted					63	50	53	54	66	60	65	57

was excreted by the rat receiving its cryolite in the food than by its pair mate receiving its cryolite in the drinking water. On an average, 65% of the ingested fluorine was excreted by the former, and 58% by the latter. It is probably significant that for the first four pairs of rats, upon which two balances were run, a considerably greater percentage of the fluorine intake was excreted for all eight rats during the last 2 weeks of feeding than during the tenth and eleventh weeks. This fact would indicate that the rate of fluorine retention decreased as the feeding period progressed.

Of the fluorine excreted, the greater portion appeared in the urine, but in this regard there was an interesting difference between the two methods of fluorine administration. In fifteen of the sixteen balance trials the percentage of the excreted fluorine appearing in the urine was greater for the rat receiving its cryolite in aqueous solution, the averages being 67 and 57, for administration in water and food, respectively.

The fact that the rats receiving fluorine in the drinking water excreted less of the ingested fluorine than their pair mates, but of what they did excrete more appeared in the urine, suggests that the difference in assimilability of cryolite dissolved in water and that mixed in the solid form (finely pulverized) with food is really a difference in the absorbability from the alimentary canal. An inspection of table 2 will show that the differences in fecal fluorine between paired rats are generally large and, with one exception (pair 4, first balance) favor the rat receiving cryolite in its food. Furthermore, the urinary fluorine excretions on intakes identical for pair mates were very nearly the same, averaging 340 micrograms for the rats receiving fluorine only in the food and 359 micrograms for the rats receiving most of their fluorine in the drinking water. We may reasonably conclude, therefore, that the difference in assimilability of fluorine ingested in water and in food is entirely due to a difference in absorbability from the intestinal tract. However, the poorer ab-

sorbability of the solid cryolite does not necessarily mean that it is only incompletely dissolved by the digestive juices. It may very well be completely dissolved, but its absorption may be hindered by the food residues with which it is mixed.

As was to be expected, the rats receiving their cryolite in the food at 4 P.M. excreted most of their urinary fluorine in the period from 5 P.M. to 8 A.M., while the rats receiving their cryolite in the drinking water at 8 A.M. excreted most of their urinary fluorine in the interval from 8 A.M. to 5 P.M. The percentages for the former rats averaged 80 for the night period, and for the latter 66 for the day period.

DISCUSSION

It is clear from these results that at a low level of fluorine intake equivalent to 10 p.p.m. of food consumed, cryolite administered in the water is retained to a greater extent than cryolite administered in the food and hence is potentially more toxic. In the preceding paper it was shown that no appreciable difference exists between the toxicity or the assimilability of the fluorine in cryolite and CaF_2 administered in the drinking water. On the reasonable assumption that fluorine as it occurs naturally in drinking water is generally present as CaF_2 insofar as it exists in unionized form, it may be concluded that for equal low dosages the fluorine in cryolite consumed with the food, as in fruit spray residues, is less assimilable than the fluorine in drinking water and hence less potentially dangerous by about 20%.

But the consumption of water is several times the consumption of food by weight. Of course the ratio will vary greatly, but a convenient and apparently satisfactory method of arriving at a normal ratio, or a ratio under usual conditions, may be based upon Adolph's ('33) estimate that "roughly a convenient liberal standard of total water intake for any mammal is 1 cc. per Calorie," the latter referring to the energy requirement. This standard agrees well with the estimated normal water intake of an average man weighing 65 kg. of 2400 cc. taken by Richter ('38) from data compiled

by Best and Taylor ('37). It agrees well with Magee's ('37) data from large numbers of institutionalized men and women, the average water intake of whom was about 2600 cc. daily in beverages, fluids and solid food. These figures would approximate the numbers of calories of food energy required by adults. In the case of children, a comparison of the cubic centimeters of water required according to McQuarrie's standards ('33) and the number of food calories required as determined by Maroney and Johnston ('37) approximate each other so closely as to suggest that Adolph's standard of 1 cc. of water per calorie of energy required is of rather general application to all ages.

Taking the fuel value of 1 gm. of dried food in the human diet at 4 calories and the water requirement at 1 cc. per calorie, the normal (or usual) ratio of water consumed to food consumed appears to be about 4 to 1. The proportion of the water intake consumed with the food will, of course, vary greatly. For purposes of calculation we may assume that one-half of the water consumed is taken as a drink and one-half in the food (Newburgh, Johnston and Falcon-Lesses, '29–'30). If twice as much water is drunk as the weight of food eaten, the critical concentration of fluorine in the drinking water defining the upper limit of safety, i.e. 1 p.p.m., would correspond to 2 p.p.m. in the food on an equal dosage basis, or considering also the relative toxicity of equal doses of fluorine in water and in food as determined in this experiment, 1 p.p.m. of fluorine in the water would be the hygienic equivalent of 2.4 p.p.m. in the food.

If all the water in the food were cooking water containing 1 p.p.m. of fluorine, then 1 p.p.m. of fluorine in a domestic water supply would be the hygienic equivalent of 4.8 p.p.m. in the food. Of course this refers to the total intake of solid food, only a small fraction of which would be contained in sprayed fruits and vegetables.

SUMMARY AND CONCLUSIONS

Experiments are reported on twelve pairs of rats designed to measure the relative toxicity of fluorine (as cryolite) when consumed in equal amounts either in food or in water. The feeding experiments were carried out according to the paired-feeding technic, and were supplemented with sixteen fluorine balance trials of 14 days each and analyses of the bones, teeth and soft tissues of all rats at the termination of the feeding period. The fluorine was fed in amounts equivalent to 10 p.p.m. of solid food; 9% of the fluorine intake was contained in the basal diet in unknown forms.

The results of the experiment seem to justify the following conclusions, under the experimental conditions above specified:

1. The method of administration of fluorine at low levels of intake whether in water or in food has no apparent effect on the rate of growth of animals. Administered in the water, however, fluorine definitely depresses the appetite of rats and induces a transient hematuria.

2. No considerable difference in the time of appearance of striations in the lower incisor teeth of rats result from the administration of fluorine in food or in water in low concentration.

3. Doses of fluorine in the food are retained to a less extent in bones, teeth and soft tissues than equal doses of fluorine in the drinking water. Considering the total retention of fluorine in the carcass, this impairment in assimilability amounts to 20%. It seems to be the result entirely of an impairment in absorption from the alimentary tract.

4. Continued ingestion of low concentrations of fluorine in food or in water by growing animals results in a smaller percentage retention in the body.

5. Considering both the difference in the usual consumption of food and of water in practical human nutrition, and the difference in potential toxicity of fluorine in water and in food, a concentration of 1 p.p.m. of fluorine in the drinking water defining the upper limit of safety, is the hygienic equiva-

lent of from 2.4 to 4.8 p.p.m. of fluorine in the total food, depending upon the proportion of the water intake that contains fluorine in the critical concentration.

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AVERAGE VALUES FOR BASAL RESPIRATORY FUNCTIONS IN ADOLESCENTS AND ADULTS [1]

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INTRODUCTION

In preparing a review of the literature concerning physiological changes in adolescence (Shock, '39), the scarcity of adequate data on basal respiratory functions became apparent.[2] Although numerous reports of oxygen consumption have been published, little information on respiratory volume, tidal volume or carbon dioxide and oxygen content of expired air is available either for adolescents or for adults under basal conditions. In such studies the maintenance of basal conditions is important since they can be most easily reproduced and thus allow comparisons between individuals. Repeated respiratory measurements have been made on a limited number of adults (Griffith et al., '29; Hafkesbring and Borgstrom, '27; Hafkesbring and Collett, '24), but such observations do not give us the probable range of values expected in normal subjects. Furthermore, it was found that with the exception of Griffith et al. ('29) in none of these studies were all the respiratory functions measured in the

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[2] Bibliographical references to this literature and to that concerned with basal oxygen consumption have been omitted from this report because of limitation of space.

same individuals. This report is presented to show the range in values of basal respiratory functions in normal adolescents and adults and to indicate changes that take place during adolescence.

METHOD

We have recorded the respiratory rate, the respiratory volume per minute, tidal volume, concentration of oxygen and carbon dioxide in the expired air, oxygen consumption and alveolar carbon dioxide tension in two groups of subjects. The first group consisted of fifty normal boys and fifty normal girls representatively sampled from normal school children, on whom the tests were begun at the ages of 11 or 12 years, and were repeated at 6-month intervals over a period of 5 years.[3] The second group consisted of forty-six normal adult males and forty normal adult females, ranging in age from 27 to 43 years. The average age of the adult males was 27.41 years (with standard deviation of 5.9 years) and of the adult females, 28.75 (S.D. = 5.8). The adults were chosen from staff members and from university students who were presumably healthy, although no systematic medical examination was given. In view of the sedentary activities of these subjects, the average values for metabolic tests may be somewhat lower than for the general population. In the adult group, we used only the tests made in triplicate on 2 successive days, although as many as sixty tests were made on each subject.

Three observations on each subject were carried out on each of 2 successive days as follows: The subject was brought by automobile from his home at 7.30 each morning without breakfast. The laboratory used was on the ground floor of the building so that activity was minimized. While the subject lay on a cot for 20-minute rest period, three determinations of blood pressure and pulse-rate were made by a trained woman assistant. At the end of 20 minutes, a Siebe-Gorman half-mask was tied over the nose and mouth of the subject.

[3] The study of these children is still in progress.

The subject was allowed 10 minutes for respiratory adjustment to the mask and then three 8-minute tests of the basal oxygen consumption were made by the Tissot open-circuit gasometer method,[4] in which the total expired air is collected and the carbon dioxide and oxygen concentration is determined by the Boothby-Sandiford modification of the Haldane technique ('20). All gas volumes were reduced to standard conditions of 0°C. and 760 mm. of mercury. Total respiratory volumes were obtained directly from the gasometer reading, and tidal air volumes were computed by dividing the total respiratory volume in liters per minute by the average respiratory rate per minute. The respiratory rate was counted twice each time for a 1-minute period, during each of the three gas-sampling periods. Samples of expiratory alveolar air were obtained by the Haldane-Priestley technic and were analyzed for carbon dioxide in the Haldane apparatus. Surface areas were derived from the height and weight of the subject by the Du Bois formula ('16).

RESULTS

The average of six determinations of each of the respiratory factors (i.e., three on each of 2 successive days) has been calculated for each subject. These values have been used in compiling frequency distributions for 100 children studied when they were 12, 14 and 16 years old, respectively, and for the ninety-six adults. The mean values with their probable errors are shown in table 1. In this table, the values for total ventilation per minute and for oxygen consumption are divided by the weight or surface area of the subject in order to take into consideration the factor of size of the individual. The reduction in variability which results from this procedure is shown in the values of the coefficient of variations which appears in parentheses in table 1.

Table 2 gives the mean differences between the respiratory functions at the age of 12 and of 14, and the age of 14 and

[4] Each subject was tested on 2 days before the actual experimental series was begun, in order to accustom him to the procedure.

TABLE 1

Mean values for respiratory functions under basal conditions at different age levels

	AGES 11.75–12.24		AGES 13.75–14.24		AGES 15.75–16.24		AGES 18.00–26.99		AGES 27.00–43.00	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Ventilation rate/min.	16.3±0.4 (21.5)	16.1±0.3 (14.5)	17.0±0.2 (14.6)	15.6±0.2 (12.5)	15.6±0.3 (14.9)	15.2±0.3 (13.5)	14.0±0.3 (15.9)	14.7±0.5 (20.8)	13.7±0.3 (14.6)	14.4±0.4 (17.9)
Ventilation volume liters/min.	4.79±0.05 (10.2)	4.54±0.07 (14.4)	5.27±0.07 (13.0)	4.86±0.06 (12.7)	5.13±0.08 (13.3)	4.21±0.08 (13.9)	5.04±0.10 (13.2)	4.45±0.11 (16.2)	5.25±0.09 (11.8)	4.63±0.08 (10.8)
Ventilation volume l./sq.m./min.	3.81±0.05 (11.1)	3.41±0.04 (10.3)	3.55±0.03 (9.5)	3.24±0.04 (12.6)	2.98±0.04 (11.1)	2.67±0.04 (12.7)	2.76±0.05 (13.3)	2.91±0.05 (11.6)	2.93±0.04 (9.2)	2.84±0.06 (14.8)
Tidal volume cc./breath	305±6 (19.4)	289±5 (17.2)	316±6 (18.3)	315±4 (13.2)	344±8 (19.7)	282±6 (16.4)	372±9 (16.2)	319±9 (19.6)	390±7 (13.7)	338±7 (13.4)
Tidal volume cc./sq.m./breath	242±4 (16.2)	216±4 (16.1)	212±3 (13.9)	208±3 (13.2)	200±3 (14.8)	177±4 (16.3)	203±5 (17.8)	202±6 (19.9)	218±4 (12.7)	202±4 (11.7)
Expired air % O_2	16.99±0.03 (1.8)	6.90±0.03 (1.5)	16.84±0.03 (1.9)	16.97±0.03 (1.7)	16.35±0.05 (2.4)	16.59±0.07 (3.1)	16.48±0.05 (2.8)	16.98±0.06 (2.4)	16.90±0.05 (1.9)	17.08±0.05 (2.0)
Expired air % CO_2	3.49±0.03 (7.6)	3.57±0.03 (5.4)	3.58±0.02 (6.7)	3.51±0.03 (7.3)	4.01±0.04 (7.6)	3.81±0.04 (8.9)	3.8±0.05 (9.7)	3.41±0.05 (9.8)	3.53±0.04 (8.9)	3.32±0.04 (8.6)
Alveolar air CO_2 tension	41.0±0.3 (6.7)	40.1±0.2 (5.4)	42.2±0.2 (5.8)	39.4±0.3 (8.3)	42.1±0.4 (7.8)	38.8±0.3 (5.8)	43.0±0.5 (5.1)	41.6±0.6 (8.1)	42.7±0.06 (8.8)	40.0±0.5 (7.8)
Oxygen consumption cc./min.	195±2 (11.7)	189±3 (13.7)	223±3 (13.1)	198±2 (11.6)	244±3 (11.5)	187±3 (10.9)	232±3 (8.4)	187±3 (9.2)	218±3 (9.5)	186±2 (8.9)
Oxygen consumption cc./kg./min.	5.15±0.06 (11.0)	4.46±0.06 (12.1)	4.65±0.05 (10.0)	3.90±0.04 (11.4)	4.13±0.04 (9.2)	3.43±0.04 (9.2)	3.43±0.05 (10.9)	3.38±0.05 (10.5)	3.39±0.05 (9.7)	3.13±0.07 (15.1)
Oxygen consumption cc./sq.m./min.	154.5±1.6 (9.6)	140.8±1.1 (7.5)	149.7±1.3 (8.9)	130.1±1.0 (7.9)	141.8±1.4 (8.6)	117.9±1.2 (7.6)	125.9±1.2 (6.8)	118.3±1.5 (8.2)	122.1±1.0 (5.6)	112.5±1.8 (10.5)
Heat production Cal./sq.m./hr.	45.03±0.46 (9.3)	40.99±0.32 (7.2)	43.46±0.35 (8.5)	37.96±0.29 (8.0)	41.13±0.38 (8.0)	34.29±0.34 (7.7)	36.57±0.38 (7.3)	34.28±0.40 (7.7)	35.44±0.28 (5.6)	32.63±0.50 (10.1)

Figures in parentheses are coefficients of variation.

and 16, in boys and girls. The significance of the age differences has been assessed by computing the ratio between the mean difference in the 100 subjects and its probable error (critical ratio). For these observations, the probable error of the mean difference was calculated from the actual distribution of differences, since each subject was measured at all three ages considered. The significant values in table 2, which indicate residual growth, appear in bold-faced type.

We have found that in boys there is an increase in the absolute minute respiratory volume (which is due to an increase in body size) between the ages of 12 and 14 years. In boys the respiratory rate decreases between the ages of 14 and 16, the tidal volume increases, the concentration of oxygen in the expired air decreases and the expired carbon dioxide increases. In girls, there is an increase in the total respiratory volume between the ages of 12 and 14 years. The changes in the composition of the expired air in girls from 14 to 16 years of age are similar to those in boys, but are not so sharply defined. The average oxygen consumption per minute increases from the age of 12 to 16 years in both sexes. However, when the oxygen consumption is measured as cubic centimeters of oxygen consumed per kilogram per minute or as calories of heat produced per square meter per hour, there is a decrease.

The statistical methods suitable for a series in which repeated observations are made on the same individuals cannot be applied to a series in which the observations are made on different individuals. For this reason, the methods used in comparing the adolescents at the ages of 12, 14 and 18 years could not be employed in comparing the 16-year-old group with the adults. This latter comparison was made by computing the probable error of the difference from the formula.

$$\text{P.E.}_{\text{Mn diff.}} = \sqrt{\text{P.E.}^2_{\text{Mn}_1} + \text{P.E.}^2_{\text{Mn}_2}}$$

The results are shown in table 3. Sixteen-year-old girls are similar to adults with respect to most respiratory functions.

Figures in parentheses are coefficients of variation.

Sixteen-year-old boys differ from adult males in that they breathe more rapidly, have a smaller tidal volume, a higher concentration of oxygen and lower concentration of carbon dioxide in the expired air, and a lower oxygen consumption or heat production.

TABLE 2

Significance of age trend in basal respiratory functions. Re-examination of same 100 children

	CHANGES BETWEEN THE AGES OF 12 AND 14 YEARS 14–12				CHANGES BETWEEN THE AGES OF 14 AND 16 YEARS 16–14			
	Girls		Boys		Girls		Boys	
	Mn diff	C.R.	Mn diff	C.R.	Mn diff	C.R.	Mn diff	C.R.
Ventilation rate/min.	−0.40	1.8	+ 0.56	1.3	−0.53	3.0	−1.64	6.8
Ventilation volume liters/min.	+0.34	6.2	+0.51	7.7	−0.52	7.0	−0.09	1.5
Ventilation volume l./sq.m./min.	−0.15	2.7	−0.29	6.5	−0.50	8.9	−0.57	15.9
Tidal volume cc./breath	+26.0	5.3	+15.0	2.5	−23.7	5.0	+30.1	6.8
Tidal volume cc./sq.m./breath	−9.97	2.8	−29.1	6.5	−24.1	7.0	−12.6	4.7
Expired air % O_2	+0.06	1.3	−0.19	5.2	−0.42	7.1	−0.51	11.2
Expired air % CO_2	−0.04	1.2	+0.12	4.7	+0.35	9.7	+0.43	12.8
Alveolar air CO_2 tension	−0.48	1.2	+1.69	5.2	+0.84	2.7	+0.04	0.1
Oxygen consumption cc./min.	+11.0	5.3	+29.1	12.5	−2.3	1.3	+23.8	8.9
Oxygen consumption cc./kg./min.	−0.57	13.5	−0.54	10.4	−0.41	8.7	−0.54	12.3
Oxygen consumption cc./sq.m./min.	−10.3	8.2	−5.6	3.6	−9.0	7.7	−7.2	5.3
Heat production Cal./sq.m./hr.	−2.88	8.3	−1.70	4.0	−2.73	7.9	−2.24	5.5

C.R. = critical ratio, calculated as $\frac{\text{Mn diff}}{\text{PE}_{\text{Mn diff}}}$

Values greater than 4.0 indicate significant differences.

TABLE 8

Significance of age difference in basal respiratory functions

	16–20				20–35			
	Male		Female		Male		Female	
	Difference	C.R.	Difference	C.R.	Difference	C.R.	Difference	C.R.
Ventilation rate/min.	+1.6 ± 0.42	3.8	+0.5 ± 0.53	0.9	+0.3 ± 0.42	0.7	+0.3 ± 0.60	0.5
Ventilation volume liters/min.	+0.09± 0.12	0.8	−0.25± 0.13	1.9	−0.21± 0.13	1.6	−0.17± 0.13	1.3
Ventilation volume l./sq.m./min.	+0.23± 0.07	3.3	−0.25± 0.07	3.6	−0.16± 0.06	2.7	+0.07± 0.08	0.9
Tidal volume cc./breath	−28.0 ±11.70	2.4	−36.8 ±11.14	3.3	−18.60±11.37	1.6	−19.5 ±11.61	1.7
Tidal volume cc./sq.m./breath	−2.9 ± 6.23	0.5	−24.8 ± 7.13	3.5	−15.2 ± 6.45	2.4	0 ± 7.03	0
Expired air % O_2	−0.13± 0.07	1.9	−0.39± 0.09	4.3	+0.41± 0.07	5.8	−0.10± 0.08	1.3
Expired air % CO_2	+0.21± 0.06	3.50	+0.41± 0.07	5.9	+0.27± 0.07	3.9	+0.09± 0.07	1.3
Alveolar air CO_2 tension	−0.85± 0.60	1.4	−2.74± 0.70	3.9	+0.35± 0.73	0.5	+1.61± 0.82	2.0
Oxygen consumption cc./min.	+11.8 ± 4.30	2.7	+0.7 ± 3.70	0.2	+13.9 ± 4.01	3.5	+0.50± 3.57	0.1
Oxygen consumption cc./kg./min.	+0.70± 0.07	10.0	+0.05± 0.07	0.7	+0.04± 0.07	0.6	+0.25± 0.09	2.8
Oxygen consumption cc./sq.m./min.	+15.9 ± 1.9	11.8	−0.4 ± 1.9	0.2	+3.8 ± 1.5	2.5	+5.8 ± 2.3	2.5
Heat production Cal./sq.m./hr.	+4.56± 0.54	8.4	+0.01± 0.52	0.2	+1.13± 0.47	2.4	+1.65± 0.64	2.6

C.R. critical ratio, calculated as $\frac{\text{Mn diff}}{\text{PE}_{\text{Mn diff}}}$

DISCUSSION

It is interesting that Griffith et al. ('29), Hafkesbring and Borgstrom ('27), and Hafkesbring and Collett ('24), found respiratory volumes which, if reduced to standard conditions of temperature and barometric pressure, fall within the range of our subjects of the same age and sex. The same groups of authors also reported carbon dioxide and oxygen contents of expired air similar to those in our series. A comparison of the respiratory volumes of our subjects with those of Trumper ('29), however, indicates that the individuals whom he studied were hyperventilating. This difference may be accounted for by the fact that our subjects were trained by repeated testings.

The average values for alveolar CO_2 tension which we have obtained are significantly higher than those reported by Griffith et al. ('29), for subjects of a comparable age. Our values are also higher than those reported by Radsma, Streef and Klerks ('33) in a group of ninety-five adults tested in the tropics. We are not prepared to say whether or not this difference can be attributed to climatic factors. Berconsky and Rossignoli ('32) also obtained slightly lower averages on a group of nineteen adult females. Our values are also slightly higher than those usually quoted in the literature from Fitzgerald and Haldane ('04), although it should be pointed out that basal conditions were not observed in tests made by Fitzgerald and Haldane. The higher values reported here are in closer agreement with the average values for CO_2 tension in arterial blood of 45.9 in fourteen males and 43.2 in seven females found by Shock and Hastings ('34).

Our average values for basal oxygen consumption of boys are, like those reported by Bierring ('31), 7 to 10% lower than the extensive series reported by Boothby, Berkson and Dunn ('36). This discrepancy may be due to the training period used in both our experiments and those of Bierring, although the number of cases in our series exceeds that of any previous studies made at this age level. In the case of girls our values are again 5 to 8% lower than most of those

reported in the literature, with the exception of the results of Benedict and Hendry ('21). However, their experiments were conducted on sleeping girls under experimental conditions that were quite different from the usual clinical procedure. At the higher age levels Stark ('32), Tilt ('30), McKay ('30) and Coons ('31) have reported values comparable to ours, although the number of subjects tested was smaller.

It is apparent from tables 1 and 2 that although total ventilation volume, total oxygen consumption and tidal volume tend to increase with age, when the factor of increase in physical size is removed these variables decrease as the subject grows older. Examination of the coefficients of variation, given in parentheses in table 1, shows also that reducing these measurements by factors involving the size of the body makes the results more homogenous. This decrease in variability as expressed by the coefficients of variation (shown in table 1) is not large in certain of the measurements, but the consistency of the trend is significant. It is evident that calculated surface area is a better measurement of body size than is body weight alone, probably because, in adults especially, body weight is influenced often by increased water and fat without an increase in metabolizing protoplasm.

In measuring the respiratory functions as described above, we have considered that values lying within $\pm$ two times the probable error of the mean ($P.E._{Mn}$) may be regarded as normal, that values lying within ± 3 $P.E._{Mn}$ are high or low normals, and that values ± 4 $P.E._{Mn}$ are definitely abnormal.

In this way it is possible to use these mean values as standards in order to determine whether or not an individual is hyperventilating. Hyperventilation will result in a) increased respiratory volume per minute or per square meter per minute, b) increased tidal volume per breath or per square meter per breath, c) lowered concentration of carbon dioxide and increased concentration of oxygen in the expired air and d) lowered alveolar carbon dioxide tension.

SUMMARY

We have tested a group of fifty male and fifty female adolescents, and a group of forty-six adult males and forty adult females; and have recorded the respiratory rate, the respiratory volume per minute, the tidal volume, the oxygen and carbon dioxide concentrations in the expired air, the oxygen consumption and the alveolar carbon dioxide tension. We have found evidence for growth changes over the adolescent period in certain respiratory functions, such as CO_2 and O_2 content of expired air, alveolar CO_2 tension and tidal volume, which have not been reported before. These measurements may serve as normal figures in the age groups from 10 to 20 years. We have pointed out some of the criteria of hyperventilation.

CONCLUSIONS

1. In boys, the minute respiratory volume increases between the ages of 12 and 14 years, due to an increase in body size.

2. In boys, the respiratory rate decreases between the ages of 14 and 16, the tidal volume increases, the concentration of oxygen in the expired air decreases, and the expired carbon dioxide increases.

3. In girls, the respiratory volume and tidal volume increase between the ages of 12 and 14 years.

4. The composition of the expired air in girls changes between the ages of 14 and 16 as it does in the boys, but the change is not so clearly defined. In boys there is a significant increase in alveolar CO_2 tension between the ages of 12 and 14 years. No significant change in alveolar CO_2 tension was found in girls.

5. The average oxygen consumption per minute increases from the age of 12 to 16 years in both sexes. In respect to body size there is a decrease in oxygen consumption.

6. Most respiratory functions of 16-year-old girls are similar to those of adult females.

7. Sixteen-year-old boys breathe more rapidly, have a smaller tidal volume, higher concentration of oxygen and lower concentration of carbon dioxide in the expired air and a lower total oxygen consumption than adult males.

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DIETARY REQUIREMENTS OF THE GUINEA PIG WITH REFERENCE TO THE NEED FOR A SPECIAL FACTOR[1]

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Study of the nutritional requirements of guinea pigs has been handicapped by the difficulty encountered in inducing these animals to eat synthetic rations. Differences of habit and anatomy make the form and consistency of the diet of far greater importance to guinea pigs than to rats. Goettsch and Pappenheimer ('31), Madsen, McCay and Maynard ('33, '35) and Hogan and Ritchie ('34) have reported experiments with guinea pigs on purified diets. The present communication deals with such experiments and with evidence which, in addition to that recently adduced by Kohler, Elvehjem and Hart ('38) indicates the existence of a distinct dietary factor required by guinea pigs.

[1] Aided by grants from the Board of Research and the College of Agriculture of the University of California, Rockefeller Foundation of New York City, and Merck and Company, Inc. The following materials were generously contributed: thiamin (betaxin) and riboflavin by Winthrop Chemical Co., Inc., vitamin B_1 concentrate by Merck and Co., Inc., wheat germ by General Mills, Inc. and brewers' yeast by The Vitamin Food Co.

EXPERIMENTAL

The diets used were as follows:

	797	800	804
Casein (alcohol extracted)[1]	22	..	..
Casein (commercial)	..	24	24
Sucrose	70	64	50
Lard	..	..	14
Salt mixture (McCollum 185)	4	4	4
Agar	4	4	4
Cod liver oil	..	2	2
Wheat germ oil	..	2	2
Yeast extract[2]	..	≏ 10 parts brewers' yeast	≏ 10 parts brewers' yeast

[1] Acid precipitated casein subjected to two 24-hour extractions with cold 66% (by volume) alcohol, followed by extraction with 95% alcohol to facilitate drying.
[2] Prepared by the method of Richardson and Hogan, '36.

Diet 797 was prepared for feeding by mixing the casein, sucrose and salts and adding the mixture gradually to boiling distilled water (200 cc. water to 1 kg. dry mixture) with constant stirring. When about three-quarters of the mixture was dissolved, one-half the agar was stirred in. After removal from the fire the remaining dry mixture was added and, finally, the remainder of the agar. The rapidly thickening diet was poured out on a slab to cool.

This diet was supplemented with:

a. Three cubic centimeters orange juice, 1.5 gm. brewers' yeast, 4 drops each of cod liver oil, wheat germ oil and ethyl linoleate daily.

b. Five cubic centimeters orange juice, 1.5 cc. yeast extract, 3 gm. brewers' yeast, 4 drops each of cod liver oil and wheat germ oil daily.

Diets 800 and 804 were prepared similarly, but the casein, salts and yeast extract were mixed separately and added to the cooling syrup.

The guinea pigs were kept on shavings. Water was always available. Except when fed by medicine dropper or stomach tube, all supplements were placed in individual feeding compartments.

In a preliminary experiment five guinea pigs, 24 to 30 days old, were placed on diet 797 plus supplements *a* (a diet found to be completely adequate for rats when one-half the quantity of supplements was given). Although the diet was eaten readily at first, the animals failed rapidly. After a month two of the guinea pigs had died and the remaining three were weak and in poor muscle tone. They ate almost none of the basic diet and were losing interest in their supplements. The fur was generally dull and rough, with loss of hair from the upper sides.

On the thirtieth day of the experiment, the surviving animals were given 30 gm. (fresh weight) of the outer leaves of lettuce. (The heavy mid-rib was not weighed nor fed). Within a few hours supplements which were virtually refused the day before were eaten greedily. Consumption of the basic ration increased greatly. Within 3 days the previously listless guinea pigs had energy enough to run about their cages and whistle loudly. They were continued on the experiment for 145 days, at which time they had gained an average of 344 gm.

There seemed two possible explanations of the results: 1) that lettuce contained a hitherto unrecognized factor necessary for guinea pigs but not for rats; or 2) that it increased one of the known factors already present above the minimal level required by this species. The lack of symptoms similar to those arising from deficiency of the known fat soluble vitamins seemed to indicate that the fat-soluble vitamins were not concerned. The animals showed no visible signs of scurvy. Goettsch and Key ('28) and Eddy ('29) have shown that 3 cc. of orange juice daily would supply sufficient vitamin C. Direct evidence that vitamin C was not concerned was afforded when the amount of orange juice was doubled on the twenty-fifth day without alleviating the deficiency. The ineffectiveness of orange juice also shows that vitamin P [4] was

[4] Zilva ('37) was unable to repeat the experiments which Béntsath, Ruszn yák and Szent Györgyi ('36) made on guinea pigs and questions the existence of vitamin P in connection with the nutrition of this species.

not the missing factor. There remained only the vitamin B complex to consider. This was supplied in the supplement by 1.5 gm. of brewers' yeast. At the start of the experiment we had intended to feed twice this quantity, but it was only with difficulty that our animals could be induced to eat even as much as 1.5 gm.

Owing to the refusal of the animals to eat larger quantities of yeast and the difficulty of training them to eat from supplement dishes, a yeast extract was substituted for yeast. In this way the supplements could be fed by medicine dropper. Guinea pigs placed on diet 797 failed just as quickly when supplements *b* were supplied as they had with supplements *a*.

In order to make certain that we were dealing with a specific need of guinea pigs whether quantitative or qualitative, not shared by rats, we performed feeding tests with rats. Six female rats placed at weaning on diet 797 plus supplements *b* in one-half the quantity given to guinea pigs, averaged 170 gm. in weight after 8 weeks. The rats were continued on the diets for 6 months and remained in good condition.

The possibility that the quantitative need for vitamin B_1 was not supplied in the yeast or yeast extract presented itself. Hence it was thought advisable to check the question directly. Six litters of guinea pigs 25 days old were segregated into five groups. They were placed on diet 797 plus supplements *b* for 10 days, at the end of which time they had lost an average of 36 gm. One of these groups was then given 30 gm. of the fresh outer leaves of lettuce. A second group received similar portions of lettuce which had been heated on open trays in a steam chamber for 1 hour at 100°C. There were no significant differences between the gains made by the guinea pigs receiving the fresh lettuce and those receiving the heated lettuce.

A third group received an aqueous extract of lettuce (LC2) = 25 gm. fresh lettuce. The extract was prepared by extracting 1 kg. freshly ground lettuce with 10 liters of distilled water at room temperature, followed by 12 hours at 2°C. The

filtered extract was concentrated under reduced pressure (temperature 35° to 40°C.) to a thick syrup (volume 20 cc.).

The guinea pigs receiving this extract made gains comparable with those receiving the fresh and the heated lettuce. The fourth group received 800 micrograms of a highly purified B_1 concentrate from rice polishings. This concentrate, when assayed at 400 micrograms with rats depleted of vitamin B_1 on the Sherman-Chase diet, produced a gain of 115 gm. (average of four females) in an 8-weeks' test period. The guinea pigs received the vitamin B_1 rich yeast extract as well.[5] The animals failed to gain.

The fifth group received 3 gm. of defatted wheat germ. The wheat germ used had been extracted with a high grade petroleum ether. Some of this same extracted wheat germ was used for the preparation of the wheat germ autolysate subsequently fed and used in other experiments in this laboratory and was known to be rich in vitamins B_1 and B_6. These guinea pigs failed to gain.

The results of these experiments are summarized in table 1.

As the feeding of considerable quantities of liquid involved much time and labor, the substitution of rations with incorporated vitamin supplements was attempted.

Diets 800 and 804 supplemented with ascorbic acid gave results identical with diet 797 plus supplements *b*. The presence of lard in diet 804 seemed without effect. The guinea pigs responded quickly when given 40 gm. of fresh lettuce daily (table 2).

Although all the evidence indicated that the factor with which we were dealing was water soluble, it seemed advisable to check this point more thoroughly. Grass (timothy lawn trimmings) was chosen as a starting material (a pilot experiment had demonstrated its growth-stimulating activity).

[5] Four rats, after a preliminary 2 weeks' depletion period on the Sherman-Chase diet, gained an average of 138 gm. in the succeeding 8 weeks on the equivalent of 1 gm. brewers' yeast as extract. The average gain with 20 micrograms of thiamin was 135 gm.

An aqueous extract was prepared by extracting 6 kg. of fresh grass with 30 liters of distilled water for 1 hour at 85° to 95°C. The extract was filtered and concentrated to a thick syrup under reduced pressure (temperature 35° to 45°C.). One cubic centimeter of this extract (GC1) was equivalent to 8 gm. of the fresh grass. This extract was fed by stomach tube to guinea pigs previously depleted on diet 800 plus 5 mg.

TABLE 1

Growth responses of guinea pigs held on purified diet 797 to lettuce, vitamin B_1 concentrate and wheat germ

TEST SUPPLEMENT	NUMBER OF GUINEA PIGS	CHANGE IN WEIGHT 10 DAYS BEFORE TEST SUPPLEMENT	CHANGE IN WEIGHT 10 DAYS AFTER TEST SUPPLEMENT
30 gm. fresh lettuce	5	−29	+20
30 gm. heated lettuce	3	−48	+17
Aqueous lettuce extract (LC2) ≏ 25 gm. fresh lettuce	2	−38	+16
800 micrograms vitamin B_1 concentrate	4	−26	−5
3 gm. defatted wheat germ	2	−53	−18

TABLE 2

Growth responses of guinea pigs held on purified diets 800 or 804 to lettuce

DIET	TEST SUPPLEMENT	NUMBER OF GUINEA PIGS	CHANGE IN WEIGHT 10 DAYS BEFORE TEST SUPPLEMENT	CHANGE IN WEIGHT 10 DAYS AFTER TEST SUPPLEMENT
804	40 gm. fresh lettuce	2	−54	+53
800	40 gm. fresh lettuce	3	−33	+39

ascorbic acid. Significant gains were made when it was fed at a level equivalent to 25 gm. of fresh grass.

An ether extract was prepared from 1500 gm. of air dried grass (moisture content 4.5%). The grass was extracted three times with 25 liters of peroxide-free ether. The extract was filtered, concentrated to about 300 cc., washed with water and finally freed of solvent. The yield was 20.3 gm. of a dark green waxy material melting above 100°C. It was diluted with ethyloleate so that 1 cc. ≏ 80 gm. of fresh grass.

This solution (GC2) melted at about 35°C., and was fed warm with a dropper. The guinea pigs failed to respond to this extract.

These results are summarized in table 3.

TABLE 3

Growth responses of guinea pigs held on diet 800 to grass preparations

TEST SUPPLEMENT	NUMBER OF GUINEA PIGS	CHANGE IN WEIGHT 10 DAYS BEFORE TEST SUPPLEMENT	CHANGE IN WEIGHT 10 DAYS AFTER TEST SUPPLEMENT
Air dried grass ≏ 30 gm. fresh grass	3	−25	+32
Aqueous grass extract (GC1) ≏ 25 gm. fresh grass	3	−11	+27
Ether extract (GC2) of grass ≏ 80 gm. grass	2	−29	−22

As the necessary factor was water soluble and relatively heat stable, the possibility existed that some part of the vitamin B_2 complex was not supplied in sufficient quantity in the yeast extract. Riboflavin and rich sources of B_6 and 'filtrate factor(s)' were then tested. Three guinea pigs depleted on diet 800 plus 5 mg. ascorbic acid for 10 days were given 50 micrograms daily of crystalline riboflavin (lactoflavin, Winthrop). Five days later two of the animals had died. Increasing the dose to 100 micrograms failed to benefit the survivor. Vitamin B_6 was supplied as a wheat germ autolysate (Birch and György, '36) and as a crude water extract of liver. Filtrate factor was given as crude liver extract and as the filtrate after the adsorption of this liver extract on fuller's earth. All extracts were administered by stomach tube.

The liver extract was made by placing 5 kg. of fresh, finely ground hog liver in 5 liters of distilled water. The mixture was heated gradually to 60°C., held at this temperature for 15 minutes, then brought to the boiling point and boiled for 5 minutes. After filtration through cheese cloth, the residue was re-extracted in the same manner using 500 cc. of distilled water per kilo of original liver. The combined filtrates

were filtered through Supercel and concentrated under reduced pressure to 1250 cc. A 750 cc. portion was concentrated to 325 cc. and this designated as crude liver extract.

Filtrate factor was prepared as follows: the remaining 500 cc. of the original liver extract was treated with two volumes of alcohol. The filtrate was freed of alcohol and adsorbed six times with 100 gm. portions of fuller's earth, shaking each time for ½ hour. The filtrate was concentrated to 200 cc.

The wheat germ autolysate was fed daily at a level equivalent to 4 gm. of the original wheat germ for 5 days without

TABLE 4

Growth responses of guinea pigs held on diet 800 to components of the vitamin B_1 complex

TEST SUPPLEMENT	NUMBER OF GUINEA PIGS	CHANGE IN WEIGHT 10 DAYS BEFORE TEST SUPPLEMENT	CHANGE IN WEIGHT TEST SUPPLEMENT 10 DAYS AFTER
50 micrograms riboflavin [1]	3	−58	−35 (1 survivor)
Wheat germ autolysate [2] ≏ 4.0 gm. wheat germ	3	−32	−7 (1 survivor)
Crude liver extract ≏ 40 gm. fresh liver	3	−26	None survived
Liver filtrate ≏ 40 gm. fresh liver	3	−23	None survived
'Synthetic' B complex	3	−31	−12 (1 survivor)

[1] Increased to 100 micrograms on fifth day.
[2] Increased to equivalent of 6 gm. on fifth day.

effect. It was then increased to the equivalent of 6 gm. daily but still failed to alleviate the deficiency. This is six to eight times the amount necessary to produce maximal growth in rats on a vitamin B complex-free diet supplemented with thiamin, riboflavin and filtrate factor, and twenty to thirty times the amount found by Birch, György and Harris ('35) to cure dermatitis and produce gains of 40 gm. in 4 weeks.

Crude liver extract and the filtrate prepared from it after the adsorption of B_1, riboflavin and B_6 by fuller's earth were both fed at levels equivalent to 40 gm. of fresh liver daily.

Neither of these preparations showed activity. In regard to B_6, Birch, György and Harris found the equivalent of 5 gm. of fresh liver daily was sufficient to support maximal growth in rats. This laboratory has found that the same liver filtrate fed at levels equivalent to 5 gm. of fresh liver daily was sufficient to support maximal growth in rats on diets lacking in filtrate factor.

Although tests of the individual members of the B complex had shown these factors to be ineffective in promoting growth in deficient guinea pigs even at high levels, the possibility existed that more than one of the factors was needed in large amount. A 'synthetic' vitamin B complex was made up so that each animal received daily 100 micrograms of thiamin (Winthrop betaxin), 200 micrograms of pure riboflavin (Winthrop lactoflavin) and assayed preparations of wheat germ autolysate equivalent to 8 gm. of wheat germ and liver filtrate equivalent to 20 gm. of liver. The feeding simultaneously of high levels of all the B factors definitely established as necessary for rats did not stimulate growth.

These results are summarized in table 4.

DISCUSSION

Where our work has been similar to that of previous investigators, the results are in general accord. Goettsch and Pappenheimer ('31) and Madsen, McCay and Maynard ('33, '35) reported that most of their animals either refused to eat their synthetic diet or died within a 3 months' period. Only two animals are reported by the first mentioned authors to have lived longer than 45 days. Madsen, McCay and Maynard had fair success when they substituted an 'AD concentrate' for cod liver oil in the diet. Eight out of ten animals survived, and 'several doubled in weight' during 70 days. This study was conducted particularly in reference to the development of muscular dystrophy which invariably occurred in guinea pigs reared on the cod liver oil containing diets. Growth on rations containing AD concentrates was superior to that of cod liver oil and dystrophy developed at a later

period. These investigators used cornstarch as the carbohydrate in their diets.

Preliminary experiments not here reported seemed to indicate that the inclusion of dextrinized cornstarch in the diet enabled guinea pigs to maintain themselves, or even gain for a variable period. Hogan and Ritchie ('34) reported that a cornstarch containing diet including 15% yeast gave rather indifferent growth when fed to guinea pigs, but when 2% tikitiki was added excellent growth was observed. In further preliminary experiments carried out in this laboratory, the inclusion of 15% Northwestern yeast led to definite weight gains. These authors attach considerable importance to the quantity of roughage in the diet. Proof that roughage does not play a part in the deficiency we have described is furnished by the fact that the deficiency could be cured by extracts.

The failure of guinea pigs to grow or maintain themselves on a purified diet, shown to be adequate for rats, seems to be due to their need for a distinct dietary factor (or factors) and not to a quantitative difference between their requirements and those of rats for the established vitamins. That fats per se play a part seems to be largely ruled out by the similar behavior of guinea pigs on diet 804 (containing 14% lard) and diet 800 (fat-low). The fat-soluble vitamins seem definitely excluded. They were supplied at high levels and more important, ether extracts of materials potent in curing the deficiency had no effect. Turning to the water soluble vitamins, the factor under consideration is distinct from vitamin C, for this vitamin was supplied in more than adequate quantities in the deficiency producing diets. Information regarding the necessity of vitamin P for guinea pigs has been conflicting, but all agree that no evidence of such a need appears in animals receiving adequate amounts of vitamin C. The fact that the deficiency appeared in animals receiving abundant C in itself shows that the missing factor cannot be vitamin P. Guinea pigs invariably failed on diets in which the vitamin B complex was supplied as yeast or yeast extract in amounts twice those shown to be adequate for rats. The

feeding of the four definitely established B factors individually in amounts greatly in excess of that required to produce maximal growth in rats, failed to save deficient animals. Feeding these same factors simultaneously at high levels was also without effect. It seems extremely unlikely that the amounts of lettuce or grass necessary to cure the deficiency could contain the quantity of B factors supplied by these concentrates. The unimpaired potency of heated lettuce is further evidence against the identity of the factor with B_1. Yeast and liver, potent sources of the entire vitamin B complex, and wheat germ, an excellent source of B_1 and B_6, are relatively low in the factor necessary for guinea pigs, while lettuce and grass, conceded to be poor in the B complex, have this essential factor in high concentration.

Since this work was completed ('37) Kohler, Elvehjem and Hart ('38) have published conclusions substantially the same as ours. Although they approached the problem from a different viewpoint and used a diet of mineralized winter milk entirely unlike the diets described here, our findings are in accord on all points. There would seem every reason to believe that we are both dealing with the same factor.

SUMMARY

1. A highly purified diet which is readily eaten by guinea pigs is described.

2. This diet failed to support guinea pigs, although supplemented with abundant orange juice and sources of the other vitamins in quantities twice those required by rats fed on this diet.

3. If small quantities of lettuce are included in the supplement, guinea pigs will grow normally for considerable periods.

4. The factor present in lettuce is also found in grass.

5. It is water soluble, fairly stable to heat (100°C. for 1 hour) and drying.

6. This factor appears distinct from the known water soluble vitamins for the latter were fed in large amounts without alleviating the deficiency.

7. The factor appears to be identical with that described by Kohler, Elvehjem and Hart.

We wish to thank Prof. Herbert M. Evans for his suggestion of the theme of these experiments and his kind interest.

ADDENDUM

After this paper was sent for publication a communication appeared by Kohler, Randle, Elvehjem and Hart ('39), who found that two unknown factors were necessary for guinea pigs when maintained on purified diets, namely, the grass juice factor and a factor necessary for the maintenance of a normal stomach lining. Grass was found to be high in the former, low in the latter.

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THE MINIMUM VITAMIN A AND CAROTENE REQUIREMENT OF THE RAT [1]

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Previous communications from this laboratory (Guilbert and Hart, '35; Guilbert, Miller and Hughes, '37) presented data on minimum vitamin-A requirements of cattle, sheep, and swine. The minimum was defined as the amount fed daily that just prevented night blindness; and the evidence indicated this amount to be a physiological minimum as regards growth, general well-being, and freedom from detectable clinical symptoms. Little or no storage occurred at these levels.

After these experiments a quantity of the same batch of oil used with cattle, sheep, and swine was saturated with CO_2 and stored at —10°C. for future reference. This cod liver oil and a sample of U.S.P. reference cod liver oil have now been utilized in parallel experiments with rats, thus enabling us to interpret previous results in terms of the reference oil. The requirement of the rat has also been studied by means of a technic as similar as possible to that used for larger animals; and the relation of the vitamin A and carotene requirements to body weight has been further tested with this species.

METHODS OF PROCEDURE

Criteria used to determine minimum requirements. Preliminary investigations showed that procedures involving testing of vision are impracticable in the rat. Differences in sensitivity to light as indicated by contraction of the pupil,

[1] This report is part of an investigation on the relation of nutrition to reproduction which became cooperative with the United States Department of Agriculture, Bureau of Animal Industry on July 1, 1929.

could not be detected in deficient animals. Wagman and Gullberg ('38) found, furthermore, by means of an infra-red photographic technic that no significant difference in rate of pupil dilation depending on vitamin-A intake could be demonstrated. We, therefore, adopted the vaginal-smear method, since aberration from normal cyclic changes is the first detectable symptom of vitamin-A deficiency thus far reported for the rat. Changes in the vaginal epithelium of the rat after vitamin-A deficiency, first observed by Evans and Bishop ('22), have served many workers as a basis for determining vitamin A. These changes are described in detail by Mason and Ellison ('35). Although most workers agree that the continued presence of cornified cells in the vaginal smear is a good index of vitamin-A deficiency, some investigators (Coward, '38) find difficulty in estimating slight responses to small doses. In the work presented here we have endeavored to eliminate this difficulty by using groups of rats which had been depleted of vitamin-A stores and which were then treated with daily doses of vitamin A, constant in respect to body weight. The test period was 60 days or longer.

Different subminimal levels produced characteristic variations from the normal vaginal-smear picture during the estrous cycle. These variations from normal were remarkably constant over long periods when the daily dose remained constant with respect to individual body weight. The rhythmic changes of the normal vaginal smear as described by Long and Evans ('22) are designated in this paper by the following abbreviations:

'O,' principally round, nucleated epithelial cells;
'corn,' cornified cells;
'leac,' cornified cells disappearing, leucocytes and epithelial cells with few if any cornified cells;
'le,' scant, 'dry' smear, consisting of leucocytes and epithelial cells with few if any cornified cells.

For convenience in reporting the data we have arbitrarily classified the daily vaginal smear records as follows: Class 1 (normal), o, corn, leac, le, o, corn, leac, le, etc.; class 2, o, corn, corn, leac, o, corn, corn, leac; class 3, corn, corn, leac,

leac, corn, corn, leac, leac; class 4, corn, corn, corn, leac, corn, corn, corn, leac; class 5, continuously cornified cell smears. As the level of vitamin-A intake was lowered, the amount and persistence of cornified cells progressively increased until they appeared continuously and masked the rhythmic changes that may have continued in the ovary. At low levels of intake, regular cycles could be discerned by less copious smears and by the appearance of leucocytes and epithelial cells at (apparently) the diestrous interval even though cornified cells did not entirely disappear. The lowest level of vitamin-A intake that maintained a normal vaginal-smear picture with a scant diestrous smear containing practically no cornified cells is considered the minimum in this investigation.

Though the classification just given is arbitrary and though only 4-day cycles are represented, there was no difficulty in classifying animals with 5- and 6-day cycles. Individual vaginal-smear records were scored according to this classification, and these were averaged to obtain a numerical expression for the vaginal-smear record for each group.

Analysis of cod liver oils. Unsaponifiable fractions of both the U.S.P. reference oil and no. 760, the oil used in the previous experiments, were analyzed at the beginning and the end of the experiments with a Hilger Vitameter A. Holmes and Corbet ('37) reported the isolation of crystalline vitamin A having a value for $E^{1\%}_{1\ cm.} = 2100$. The following tabulation gives the vitameter determinations on both oils, using first $E^{1\%}_{1\ cm.} = 1600$, the value assigned by Carr and Jewell ('33), and then $E^{1\%}_{1\ cm.} = 2100$, the value reported for crystalline vitamin A. The figures (1) and (2) following the designation of the oil represent values obtained during the earlier and the present experiments respectively.

COD LIVER OIL	$E^{1\%}_{1\ CM.}$	PER CENT VITAMIN A $E^{1\%}_{1\ CM.} = 1600$	PER CENT VITAMIN A $E^{1\%}_{1\ CM.} = 2100$
U.S.P. Ref. (1)	1.31	0.082	0.062
U.S.P. Ref. (2)	1.32	0.083	0.063
No. 760 (1)	1.32	0.083	0.063
No. 760 (2)	1.35	0.084	0.064

Judging from the results, the potency of oil no. 760 did not change during the various experiments, and the extinction coefficients of this and the U.S.P. reference oil were the same. Our value for the reference oil, $E^{1\%}_{1\,cm.} = 1.32$, is exactly that found by McFarlan, Reddie and Merrill ('37). Considering the reference oil to contain 3000 I.U. per gram, the conversion factor would then be 2270. This E value is also within the range obtained by various investigators and reported by Irish ('36), Wilkie ('38) and Hume and Chick ('35). Since the physical constant of Holmes and Corbet ('37) is based upon a more highly purified form of vitamin A, we have used the value 0.063% vitamin A for both oils. Dosage is calculated and former results are revaluated on this basis. The cod liver oils were diluted with cottonseed oil for feeding.

Carotene. The crystalline carotene was obtained from the S.M.A. Corporation and was from the stock used in the previous experiments with sheep and swine. It contained about 88% beta and 12% alpha carotene. A stock solution was made containing 0.31%[2] carotene in cottonseed oil. From this stock for daily use, was prepared a more dilute solution containing 0.05 microgram of carotene per milligram of oil. Only enough dilute solution to last about 1 week was made at one time. Colorimetric analysis was made at frequent intervals throughout the experiments, both on the stock solution and on the solution used for feeding. The stock solution was kept in the dark at 15°C.

Dosing of rats. The oil solutions containing vitamin A or carotene were administered daily per os from a 2 cc. hypodermic syringe fitted with a no. 26 needle. The tip of the needle was ground off squarely, and each tip calibrated for delivery of one drop of oil. Repeated weighings of drops of oil showed that the variation in the amount delivered did not exceed ±5%. All levels of dosage were maintained constant with respect to body weight. The rats were weighed every 5 days, and the dosage calculated for the next 5 days for each weight.

[2] Colorimetric determination using dye standard matched against pure beta carotene M.P. 184°C.

Animals used and basal diet employed. During the last 10 days of lactation the stock colony diet of the mother rats was replaced by the vitamin A-free basal diet. The young female rats used in the experiments were placed on the basal diet when they were weaned at 21 days of age. The composition of this diet was as follows:

	%
Cornstarch	67
Casein, heat treated, 7 days at 100°C.	20
Brewer's yeast, irradiated	5
Cottonseed oil	5
Salt mixture no. 351 (Hubbell et al., '37, p. 276)	3
	100

RESULTS

Depletion time. Vitamin-A therapy was instituted when individuals had shown continuous cornification of the vaginal smear for 5 successive days. Control rats receiving no supplement at this time were never observed to return to a normal smear. We refer to the beginning of this period of

TABLE 1

Summary of 200 rats on basal diet from weaning to time of vitamin-A depletion

	WEIGHT AT 21 DAYS	VAGINAL SMEAR BECAME CONTINUOUSLY CORNIFIED	
		Age	Weight
	gm.	*days*	*gm.*
Mean	46.0	53.1	165
Standard error	±0.32	±0.32	±0.45
Coefficient of variation	9.8%	8.7%	3.9%

continuous cornified smear as the time of depletion. Table 1 summarizes the results with 200 rats up to the time of depletion as defined above.

Fifteen untreated controls continued to gain weight for an average period of 27 days after the appearance of continuous cornified smears and reached an average weight of 200 gm. Subsequently they developed other typical symptoms of vitamin-A deficiency, declined in weight and died.

Minimum vitamin A and carotene requirement of the rat. Table 2 presents the data on all rats used in these experiments. The minimum level to maintain normal vaginal smears in all rats in a group was, for oil no. 760, between 4.6 and 5.3 μg. per kilogram body weight. Since five out of six rats

TABLE 2

Effect of varying levels of vitamin A and of carotene on vaginal smears of rats

SOURCE OF VITAMIN A	NUMBER OF RATS IN GROUP	NUMBER OF DAYS ON TEST	DAILY INTAKE, VITAMIN A OR CAROTENE, μG. PER KILOGRAM BODY WEIGHT	INDIVIDUAL VAGINAL SMEAR SCORES	AVERAGE VAGINAL SMEAR SCORE
C.L.O. no. 760	6	65	3.0	444333	3.5
	6	73	3.8	322211	1.8
	6	62	4.6	211111	1.2
	6	60	5.3	All normal	1.0
	6	60	6.1	All normal	1.0
U.S.P. ref. oil	6	65	3.0	443333	3.3
	6	73	3.8	221111	1.3
	7	67	4.6	All normal	1.0
	6	60	5.3	All normal	1.0
	6	60	6.1	All normal	1.0
Carotene	14	70	6	44444444333322	3.4
	15	70	8	443333222222222	2.5
	12	104	10	333322222211	2.2
Carotene + bile salts	9	104	10	332221111	1.8
Carotene	15	70	11	3222222222211111	1.8
	13	104	13	322222111111	1.6
	15	70	14	2222222211111111	1.5
	12	104	15	222111111111	1.2
	6	53	20	All normal	1.0
	6	53	40	All normal	1.0
	6	53	80	All normal	1.0
	5	53	200	All normal	1.0
	6	53	400	All normal	1.0
	6	53	600	All normal	1.0

on the lower level were normal, the minimum level is probably nearer 4.6 than 5.3. In terms of the U.S.P. reference oil the minimum level was between 3.8 and 4.6 μg. per kilogram body weight. Thus the reference oil showed slightly greater potency, although the extinction coefficients were practically

equal. Based on the average group scores, 4.7 and 4.1 μg. per kilogram body weight may be taken as the minimum levels for oil no. 760 and the reference oil respectively. Since the dilutions and amounts of oil fed were the same, the value of oil no. 760 may be calculated as 87% that of the reference oil or 2600 U.S.P. units per gram. At each level of dosing, the average vaginal smear score was slightly lower for the U.S.P. reference oil than for oil no. 760, a fact which probably means that there was more irrelevant absorption at the vitamin-A wave length in the latter oil. On the basis of the reference cod liver oil, the minimum level is 18 to 22 I.U. per kilogram body weight per day.

Table 2 shows that the minimum level in terms of carotene lay between 15 and 20 μg. per kilogram body weight. As Greaves and Schmidt ('35) have demonstrated, desoxycholic acid plays an essential role in the absorption of carotene in the rat. To test the effect of additional bile salt in normal animals, nine rats were given 1% desoxycholic acid in their basal ration and were held during the test period of 104 days on a 10 μg. per kilogram carotene level. Table 2 shows a slightly improved utilization of carotene in these rats.

At autopsy, about 45 days after depletion, no storage was found in the livers of rats receiving the higher levels of vitamin A. On the other hand, some storage was found in rats on levels of 4 to 6 μg. per kilogram that were autopsied after 90 days or more of vitamin-A dosage. Baumann et al. ('34) found no storage after 4 weeks' dosage at approximately twice our minimum level in rats that had been depleted to the stage of incipient xerophthalmia. Approximately four times our minimum level resulted in storage under like conditions. This apparent difference in minimum level to produce storage probably resulted from differences in the degree of vitamin-A depletion of the rats before dosage was commenced and in the length of period of dosage before autopsy was made. We could not demonstrate the storage of carotene in the livers of rats treated with carotene until a daily dose of

80 μg./kg. was reached. This represents four to five times the minimum level to produce a normal vaginal smear.

There was no significant difference in weight gains in any of the various levels of vitamin A or carotene dosage. Control rats, however, before showing weight losses, attained the weight at which the growth curve normally flattens out. The conditions of these experiments are therefore not critical as regards minimum levels to support normal gains. The data do show, however, normal gains at the minimum level that prevented any abnormal degree of vaginal cornification.

Chemical analyses were made upon the carcasses of a number of rats from each group at the end of the test period on various levels of vitamin A. These were compared with analyses of controls taken at the beginning of the test period when vitamin A was depleted. The results revealed no significant differences in the percentage composition of the tissues.

Despite some variation in the character of vaginal smears between animals on the same level of vitamin-A intake, the vaginal-smear picture of individual rats remained remarkably constant after a period of 10 to 20 days following the institution of vitamin-A therapy.

Effect of large single doses of vitamin A. Rats depleted to such a degree that they exhibited 5 days' continuous cornified smears were given single doses at the rate of 150 and 300 μg. per kilogram body weight respectively. The time elapsing before vaginal cornification reappeared was recorded. Several such tests were made on each individual. The results were as follows:

Number of trials	*Rate of dosage μg. per kg.*	*Number of days from dosing to reappearance of vaginal cornification*
15	150	21.6±0.8
10	300	27.6±1.7

As compared with daily dosing, the smaller of the single doses was about 70% efficient, and the larger about 45%. In a series of experiments by Greaves and Schmidt ('36) female rats which had been depleted of vitamin A as judged from the

vaginal-smear picture were given a large single dose of vitamin A (375 units). Their rats varied from 117 to 181 gm. when the dose was given and received protection for 23 to 24 days. This dose is two to four times as great as in our experiments yet the period of protection was about the same. We can offer no explanation for this discrepancy except that the conditions of our experiments were probably not the same as theirs. It seems evident again from these experiments that the amount of vitamin A which can be absorbed and stored from one single dose, is limited. These results, which again confirm the findings of Baumann et al., emphasize the importance of daily feeding of small doses in determining the minimum level of vitamin A and carotene.

DISCUSSION

The minimum vitamin-A intake required to prevent vaginal cornification in the rat was found to be about 4.1 μg. per kilogram for U.S.P. reference cod liver oil and about 4.7 μg. per kilogram for oil no. 760 on the basis of vitameter analyses. These results, converted to biological units on the basis of the reference oil, show the minimum requirement to be 18 to 22 I.U. per kilogram body weight. This coincides with the data presented by Guilbert and Hart ('35) and by Guilbert, Miller and Hughes ('37) when similarly converted. The minimal carotene requirement for the rat was between 15 and 20 μg. per kilogram, an amount slightly lower than that found for domestic animals.

The uniformity of response to definite levels of intake over periods during which body weight and stage of maturity changed significantly, together with the close agreement with previous data, confirms the relation of requirement to body weight.

Recognition and application of this fundamental principle to biological test procedures should still further reduce variability of results. Adaptation of the method of procedure employed in these investigations has promising possibilities for routine biological testing. With a standardized procedure

the test period could be reduced to 4 weeks, and the same animals could be used repeatedly. The method has an advantage over the vaginal smear methods previously used in that responses to small doses can be measured. The advantage over the growth procedure apparently rests on the fact that only earliest manifestations of deficiency are involved, in contrast to more drastic pathological changes that may be present when growth ceases. These conditions undoubtedly contribute to variation in growth response. The disadvantage is that the method is somewhat laborious.

SUMMARY

By means of a vaginal-smear technic and a daily dosing of rats at definite levels with respect to body weight, the minimum level to prevent vaginal cornification was found to be 3.8 to 4.6 μg. or 18 to 22 I.U. per day of vitamin A per kilogram body weight.

Similarly, the minimal carotene level was between 15 and 20 μg. per kilogram body weight. No detectable storage occurred until a level of 80 μg. per kilogram body weight per day was given. Large single doses were less efficiently utilized than daily doses, the efficiency decreasing with increasing size of dose.

Simultaneous administration of bile salts with carotene slightly increased its efficiency.

The results confirmed previous data on the relation of vitamin-A requirement to body weight.

The application of this principle to biological testing in general is discussed, together with the adaptability of the vaginal-smear technic used in these experiments for routine biological testing.

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CONDITIONS AFFECTING THE CONTENT OF CHICK ANTIDERMATITIS VITAMIN IN YEAST [1]

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Yeast is an important source of the B vitamins. It is now recognized as a material rich in thiamine, riboflavin, nicotinic acid and for years has been included in many experimental diets as a source of one or more of these essentials. While yeast may be a good source of the B vitamins, it is apparent that different yeasts vary greatly in their vitamin content. The well-known difference between brewers yeast and bakers yeast is an example in point. The former is usually five times as rich as the latter in vitamin B_1. Likewise some commercial bakers yeasts contain twice as much B_1 as other samples.

The most important factor affecting the B_1 content seems to be the kind of medium in which the yeast is propagated. In a previous publication (Pavcek, Peterson and Elvehjem, '37) it was shown that bakers yeast contained about 10 international units per gram of dry matter when grown in a grain wort medium, but only 5 international units when grown in a molasses-salts medium. The figure fell to about 3 international units when the yeast was produced in a synthetic medium, glucose-salts. These same batches of yeast have now been assayed for their content of the chick anti-dermatitis factor and the results are given herein. Very few assays of yeast have been made with respect to this factor and none in

[1] Published with the approval of the director of the Wisconsin Agricultural Experiment Station.

relation to the medium upon which the yeast was grown. The recent demonstration of the relation of pantothenic acid to the chick antidermatitis factor increases the significance of these results.

EXPERIMENTAL

Production of yeasts. The conditions under which the yeasts were grown, such as composition of medium, methods of aeration, period of fermentation, etc., have been described in detail (Pavcek, Peterson and Elvehjem, '37), and hence will not be repeated here. The procedures generally used in commercial practice were followed, and the yields were approximately those obtained in the industry. Likewise the vitamin B_1 content of the experimental yeasts were essentially the same as those of commercial yeasts when similar methods of propagation were employed. We feel, therefore, that the yeasts represent what may be expected when well-recognized methods of production are followed.

Chick assay. Day-old white Leghorn chicks were fed the basal ration, 241H (Mickelsen, Waisman and Elvehjem, '38) which contains all the vitamins known to be required by this species, except the antidermatitis factor. Four chicks were used in a group for each assay. The dried yeasts were added at various levels to the basal ration. If necessary, the assays were repeated until a level was found at which protection was obtained and another at which symptoms of dermatitis appeared in one or more members of the group. The minimum protective level, therefore, lies between these two figures. As a check on the procedure both positive and negative controls were run. The negative controls usually developed severe dermatitis and most of them died in from 3 to 4 weeks. When an occasional bird survived longer than this, the gain in weight was never over 50 gm. in 5 weeks. The feeding period extended over 6 weeks, but the gain in weight made in 5 weeks was taken as most suitable for record. Because of possible variations both in yeasts and in chicks, a considerable number of duplicate batches of yeast were grown and assays repeated.

Duplicate assays on the same batch of yeast agreed very well and chick assays of different batches grown on the same medium showed less variation than might be expected.

Data. Assays of twenty-three experimental batches of yeasts are given in table 1. Those grown in grain-wort were richest in anti-dermatitis factor, the molasses yeasts were next in order and the glucose-salts batches contained the least

TABLE 1

Assay of laboratory-grown yeasts

NUMBER	YEAST	LEVEL FED	AVERAGE GAIN IN 5 WEEKS	NUMBER OF CHICKS SHOWING SYMPTOMS
		%	*gm.*	
Control, 3 lots		0	48[1]	12
Series I. Yeasts grown on glucose-salts medium				
131	S. cerevisiae	4	71	3
131	S. cerevisiae	6	94	0
405	S. cerevisiae	4	65	3
405	S. cerevisiae	6	94	0
212	S. logos	2	88	3
212	S. logos	4	83	1
305	W. anomala	4	54	2
312	E. vernalis	6	122	0
Series II. Yeasts grown on molasses-salts medium				
101	S. cerevisiae	2	125	1
101	S. cerevisiae	4	165	0
406	S. cerevisiae	2	62	3
406	S. cerevisiae	4	112	0
304	W. anomala	4	52	3
313	E. vernalis	4	82	4
Series III. Yeasts grown on grain wort medium				
208B	S. cerevisiae	2	108	2
208B	S. cerevisiae	4	122	0
400	S. cerevisiae	1	49	4
400	S. cerevisiae	2	123	1
400	S. cerevisiae	4	172	0
210B	S. logos	2	117	0
210B	S. logos	4	141	0
302	W. anomala	2	91	0
302	W. anomala	4	135	0
311	E. vernalis	2	64	1

[1] Average for two chicks. Other ten died before the end of 5 weeks.

amount of the factor. The protective levels for the three groups were approximately 2, 4 and 6%. Some variations from these figures were obtained, but these were not greater than would be expected from different batches of yeast and from different lots of chicks. For example: S. logos (no. 212) in the glucose-salts series seemed somewhat better than the other yeasts in this series and gave almost adequate protection at a 4% level. In the molasses group, W. anomala (no. 304) and E. vernalis (no. 313) proved inadequate at 4%.

TABLE 2

Assay of commercial yeasts

NUMBER	YEAST	LEVEL FED	AVERAGE GAIN IN 5 WEEKS	NUMBER OF CHICKS SHOWING SYMPTOMS
		%	*gm.*	
401	Bakers B	2	143	0
401	Bakers B	4	135	0
402	Bakers A	1	115	3
402	Bakers A	2	172	0
402	Bakers A	4	216	0
407	Bakers F	2	112	1
407	Bakers F	4	126	0
316	Brewers P	2	115	2
316	Brewers P	4	150	0
403	Brewers L	2	112	0
403	Brewers L	4	185	0
404	Brewers C	1	52	3
404	Brewers C	2	127	0
404	Brewers C	4	187	0

However, one batch of S. cerevisiae (no. 101) gave very good growth at 2% and only one of the four chicks in the group showed symptoms of dermatitis. The figure, 2%, is perhaps somewhat low for grain-wort yeasts. Slight symptoms of dermatitis appeared in three cases at this level. At 4% there was no evidence of dermatitis, and growth was exceptionally good.

Assays of commercial yeasts are given in table 2. Most, if not all, were grown on a wort medium and the protective level was 2%. This is the same figure as was found for the labora-

tory yeasts, grown on a wort medium. No difference is manifest between bakers and brewers yeasts in respect to the chick factor. This result is in marked contrast to the ratio between the two with respect to vitamin B_1. Brewers yeast usually contains several times as much vitamin B_1 as bakers yeast.

The figure for grain-wort yeasts and commercial yeasts is about the same as that for animal tissues, richest in this vitamin. Liver and kidney protect at a 2% level but some tissues e.g., lean beef, must be fed at a 30% level (Waisman, Michelsen and Elvehjem, '39).

DISCUSSION

From the recent work of Woolley, Waisman and Elvehjem ('39) it appears that the chick anti-dermatitis factor is pantothenic acid. This compound has been extensively investigated by Williams and associates ('33, '34, '39) who found that yeast growth is greatly stimulated by additions of pantothenic acid to the medium. Although yeast growth is stimulated by pantothenic acid, the data reported here show that pantothenic acid is also a product of yeast growth. The yeast crop on the glucose-salts medium ranged from 353 gm. for batch 305 to 1037 for batch 131. The inoculum, a wort-grown yeast, was equivalent to 50 gm. of dry yeast. Hence the multiplication of cells was from seven- to twentyfold, while the potency decreased only to one-third of the original value. The innoculum, therefore, contained only from 15 to 40% as much pantothenic acid as the yeast crop. A similar situation exists with respect to vitamin B_1, which may be both a stimulant and a product of yeast growth.

SUMMARY

The amount of chick anti-dermatitis vitamin (pantothenic acid) in yeast varies with the medium in which the yeast is grown. Yeast grown in grain wort gave protection when fed at a 2% level, molasses yeast at 4% and glucose-salts yeast at 6%. Commercial bakers and brewers yeasts also protected at

the 2% level, which is about the potency of animal tissues richest in this vitamin.

The authors are indebted to P. E. Hubanks for assistance in the feeding work, and to N.Y.A. for funds in payment of his services.

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INFLUENCE OF MASSIVE DOSES OF VITAMIN B_1 ON FERTILITY AND LACTATION [1]

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Following the numerous reports on the toxic effects of massive doses of vitamin D, it was logical for nutritional investigators to be interested in the accumulation of evidence on the possible existence of other hypervitaminoses. For some years it was planned to study vitamin B_1 from this standpoint but our studies on the biochemistry and pathology of deficiency diseases did not permit following this line of research. The report, however, of Perla ('37) that vitamin B_1 in amounts equivalent to forty times the maintenance requrement produced toxic effects as evidenced by inability of rats to rear their young successfully, stimulated this investigation, which was begun in January of 1938.

Perla used a stock diet consisting of 15 gm. per day of a basic mixture of hominy 100 parts, rolled oats 15 parts, fine meat and bone 25 parts, salts 1½ parts, and dried milk 16 parts, to which was added a few drops of cod liver oil, 0.3 gm. of wheat germ and 0.3 gm. of crude Fleischman's brewer's yeast per rat. He found that supplementing his stock diet with Mead Johnson's brewer's yeast equivalent to 50 I.U. of vitamin B_1 per animal per day resulted in a disturbance in lactation in the first generation which was accentuated in the second generation. The effect was more pronounced in

[1] Research paper no. 620, journal series, University of Arkansas. This is paper XXV in the series Dietary Requirements for Fertility and Lactation.

animals fed a vitamin B_1 concentrate (adsorbate) among which still-births were also common. Synthetic vitamin B_1 was somewhat less toxic than the vitamin B_1 adsorbate but similar interference with lactation occurred in the second generation.

While the work initiated in our laboratory was in progress the monograph on vitamin B_1 (thiamin)[2] by Williams and Spies ('38) appeared in which they criticized the findings of Perla. Since Perla injected the synthetic vitamin B_1 subcutaneously, they state: "The disturbances of very young litters for purposes of daily injections seems a questionable practice which might well lead to the abandonment of the young by the mothers." Furthermore, they refer to experiments of Ammerman and Waterman who administered 80 to 1000 μg. of thiamin daily to each rat as a supplement to a stock diet, which was continued for three generations, and reproduction was perfectly normal. Since no reference is given to this work in the bibliography, the work quoted probably represents unpublished data. Without knowing the composition of the stock diet and in the absence of experimental data, it is impossible to evaluate the investigations of Ammerman and Waterman. But at least it appears that we have conflicting results on the influence of massive doses of vitamin B_1 on lactation efficiency when carried on for several generations. It is therefore, all the more pertinent that the results of this laboratory be reported at this time.

Since 1920 we have employed the Wistar strain of albino rat and have successfully reared our animals on the following stock diet: whole wheat, 27; rolled oats, 26; yellow corn, 25; linseed meal, 15; commercial casein, 5; cod liver oil, 1; NaCl, 0.5; and $CaCO_3$, 0.5. This diet is supplemented 6 days a week with 5 cc. of fresh cow's milk per animal until breeding when it is increased to 10 cc. and during lactation, when the young begin eating, to 15 cc. daily. Each animal also receives 15 gm. fresh lettuce once weekly. On such a ration we raise 95 to 98% of our young, the mortality being less

[2] Vitamin B_1 and thiamin are used interchangeably in this paper.

than 5%, and very seldom do we encounter a case of sterility. When we attempted to remove either the supplementary lettuce or milk from this stock diet, the infant mortality increased to over 50%. Removing both the milk and lettuce increased the infant mortality to 60% accompanied by sterility. Therefore, as a starting point in this research, our stock diet supplemented with milk and lettuce was employed.

EXPERIMENTAL

The rats in this study were divided into four groups: A) each animal received 10 μg. thiamin daily; B) each animal received 100 μg. thiamin daily; C) each animal received 200 μg. thiamin daily, and D) each animal received 400 μg. thiamin daily. The thiamin used was the pure crystalline product of Merck.[3] The results are summarized in table 1.

Group A. Since 10 μg. of thiamin was found sufficient as a curative dose for even the most marked cases of polyneuritis associated with convulsions and at the same time was accompanied by very good growth, it was at first thought to use this daily amount as a standard of comparison. This was hardly justifiable, since the stock diet furnished in itself liberal amounts of vitamin B_1. On this daily dose fertility and lactation was perfectly normal for two generations. Therefore, it was considered advisable to use a large dose during the third generation. The daily dose was increased to 200 μg. thiamin daily. Toxic effects then became apparent. The lactation efficiency dropped from 95 to 41%; also, one female was sterile and post-mortem examination showed resorption of twelve remaining embryos. The resorption was indistinguishable from vitamin E deficiency.

Group B. No toxic effects were encountered in the first generation on the 100 μg. thiamin daily dose. In the second generation, however, two females were sterile, but the two females that were fertile reared their young successfully. In the third generation when the daily thiamin dose was in-

[3] We wish to express our appreciation to Merck and Co. for the thiamin donated for this work.

creased to 400 μg., five females completely failed to rear their seven litters and one was sterile. In other words, infant mortality was 100%. Almost invariably the litters died the first few days of the nursing period and canabalism was very marked, which was associated with a loss of the maternal instinct.

Group C. On the daily dose of 200 μg. thiamin, two mothers out of six became sterile in the first generation. Since only

TABLE 1

Influence of massive doses of vitamin B_1 on fertility and lactation

Group Vitamin B_1 daily dose	Generation	Females	Litters	Young born	Young allowed to be reared	Young weaned	Per cent young weaned	Remarks
A								
10 μg.	First	4	4	37	24	23	96	
10 μg.	Second	6	6	49	32	30	94	
200 μg.	Third	6	6	59	36	15	41	One female was sterile after 161 days of mating. Another female resorbed twelve embryos.
B								
100 μg.	First	4	4	36	24	24	100	
100 μg.	Second	4	2	21	12	12	100	Two females were sterile.
400 μg.	Third	6	7	64	42	0	0	One female was sterile after 110 days of mating.
C								
200 μg.	First	6	4	43	24	24	100	Two females were sterile.
200 μg.	Second	2	2	12	12	12	100	
600 μg.	Third	6	2	25	12	0	0	Four females were sterile 120 days after mating. One female resorbed fourteen embryos.
D								
400 μg.	First	4	6	61	34	32	94	
400 μg.	Second	6	6	69	36	30	83	
800 μg.	Third	6	14	147	78	10	12	

two mothers were available for the second generation, the insufficient number of animals are not a criterion for the fertility and lactation performances. In the third generation on the 600 μg. daily thiamin dose, however, out of six females four were sterile, one with a clear case of resorption indistinguishable from vitamin E deficiency and the other two that were fertile entirely failed to rear their litters, death of the young having occurred during the first few days of lactation.

Group D. It is surprising that on the 400 μg. daily thiamin dose, fertility and lactation should have proceeded normally during the first two generations, but lactation was a pronounced failure during the third generation on the 800 μg. daily vitamin B_1 dose, the infant mortality being 88%.

Growth in all instances with the thiamin supplements was much greater than on the stock diet alone.

At no time throughout this study was the regular stock diet with its supplements of milk and lettuce changed, and all the daily thiamin administrations were given orally in solution in Petri dishes, which all the animals eagerly consumed. The mothers were tame at all times, although when by virtue of the large doses of thiamin they were unable to lactate, they became disinterested in the welfare of their young and devoured them, a phenomenon characteristic of the experimental rat when there is a deficiency of an essential factor in the diet.

While Perla[4] on his stock diet began to encounter toxic symptoms on lactation in the first generation, the injurious effects on lactation of massive doses of thiamin, supplementing our stock diet were not manifested until the third generation. The injurious effects on fertility, however, are apparent in the first generation. Beginning with a 400 μg. thiamin daily dose the physiological mechanism of milk secretion

[4] Perla's recent findings ('39) that a daily allowance of 2 mg. manganous chloride counteracts the toxicity of large doses of thiamin lactation is of considerable interest. It would link vitamin B_1 metabolism with manganese, as vitamin D is associated with calcium and phosphorus metabolism. Also the giving of manganese salts might be indicated in various types of neuritis or other diseases where large doses of this vitamin are prescribed.

collapses. Out of 132 young allowed to be reared on 400 to 800 μg. daily doses of thiamin, only ten young were reared. Since this work was completed, Perla ('39) in a preliminary report also cites progressive decrease in fertility on daily supplements of 30 I.U. of vitamin B_1.

The fact that resorption of the foetus during gestation was observed in vitamin A deficiency (Sure, '28) and in toxic doses of vitamin B_1, we can no longer consider such phenomenon as specific for vitamin E deficiency.

The question arises: What daily dose of thiamin did the stock diet with its supplements provide? Before such calculation can be made it is necessary to know the thiamin content of each of the constituents of this diet expressed as micrograms per gram. From the thiamin content of foodstuffs given by Williams and Spies in their monograph ('38) whole wheat, rolled oats and yellow corn contain 4.72, 3.00, and 2.5 μg. per gram respectively. No figures are given for linseed meal nor for commercial casein. But it is estimated that linseed meal may contain 3 μg. per gram and commercial casein 2.0 μg. per gram. The latter should be a liberal figure, since milk powder contains only 2.5 μg. per gram. Lettuce contains 0.2 μg. per gram fresh tissue, and whole milk 0.45 μg. per gram. From such figures it is estimated that 100 gm. of our stock diet will contain about 328 μg. thiamin. A growing animal eating 15 gm. of such feed daily will then receive approximately 50 μg. of thiamin. A lactating rat which is eating 25 to 30 gm. of feed and drinking 10 to 15 cc. of fresh cow's milk, will consume 100 to 110 μg. thiamin daily. Since sterility was encountered in the second generation on 100 μg. of thiamin, supplementing our stock diet, which in itself provided an equal dose of this vitamin, it appears that twice the daily intake of thiamin furnished by our stock ration interferes with reproduction. Since 2.5 μg. thiamin daily per rat will prevent polyneuritic symptoms and loss of weight, eighty times the maintenance dose of thiamin is toxic from the standpoint of reproduction and 120 times the maintenance dose definitely toxic for lactation. Furthermore, studies just

completed on lactation with purified diets (using crystalline thiamin, riboflavin, B_6, choline, nicotinic acid and W factor as components of the vitamin B complex) indicate the daily requirement of vitamin B_1 to be 120 μg. In other words, three times the requirements for lactation (as found in short time transfer experiments from stock diet to synthetic diets) are definitely toxic in the third generation animals fed our stock diet. Just what manganese may do to counteract such toxicity (Perla, '39) is being checked at this time. When it is considered that Bills ('30) found that vitamin D (activated ergosterol) administered to rats in doses 100 times greater than the minimum antiricketic level did not produce a toxic effect on growth and reproduction, and that 1000 times the overdosage was just perceptibly harmful, and that it took a 4000 times overdosage to be definitely injurious, the possible toxic effects of vitamin B_1 or thiamin appear quite serious. It is doubtful, however, since vitamin B_1 is quite deficient in the American diet (Sure, '33; McCollum, '35) whether we will encounter injurious effects from an overdosage. However, we must bear in mind that large doses of thiamin for long periods of time may affect the pregnant and nursing mother.

SUMMARY

A daily dose of 100 μg. of thiamin results in female sterility in the second generation. A daily dose of 200 μg. of thiamin produces toxic effects in lactation in the third generation. A daily dose of 400 μg. of thiamin results in entire failure in lactation in the third generation.

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BASAL METABOLISM OF CONNECTICUT STATE COLLEGE STUDENTS

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THREE FIGURES

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A study of the basal metabolic rate of women in some of the nutrition classes at the Connecticut State College was started in 1933, but most of the work was done from 1935 to 1938. After 1933, the study was not confined to students in nutrition classes and included both men and women students.

Our original aim was to get some idea of the average basal rate of the students and to see how these averages compared with the averages of college students in other parts of the country. As work progressed the range in basal rates or the variations shown by individuals became a matter of considerable interest.

Basal metabolism studies by Hitchcock and Wardwell ('29) on college women have indicated that there is a cyclic variation in the basal metabolic rate. Studies by Tilt ('30, '35), Mason and Benedict ('34) and McKitterick ('36) have indicated that climate and altitude are factors that may effect basal rate. The observations of Coons and Schiefelbusch ('32) on type and quantity of food, of Hackett ('31) on coffee drinking and of Dill, Edwards and Forbes ('34) on smoking indicate that these factors may have some effect on the basal rate. Henry ('30) considers that overactive states and nervous or emotional strain may produce effects on the basal rate.

The Connecticut State College is situated at Storrs, Connecticut, a village 640 feet above sea level. The temperature range for 1937 was 6° to 96°F. with an average of 49.09°.[1] The climate is considered as good as may be found in the southern New England hills. The winters are not very cold and the summers are fairly cool. The village is 8 miles from the nearest city so is as nearly rural as any eastern Connecticut village.

One hundred and ten college women and fifty college men, 17 to 27 years of age are included in this study. These were selected from a large group and were all healthy and normal in every respect as far as could be determined by college health records or in any other way. The studies were carried on from September to June each year except 1937–1938 when a few students in the summer session were given tests.

The tests were made with a Benedict-Roth respiration calorimeter, which was kept in good condition and tested as to accuracy several times each year.

The students came to the basal metabolism room before breakfast and rested 15 minutes to ½ hour, depending upon the exertion necessary in getting to the room. Most of the women students came from the same building and came soon after awakening and without fully dressing. The men came from dormitories within 5 minutes walk of the basal metabolism room. Every student was questioned as to whether he had slept well and had slept the full number of hours that were customary for him. This varied from 7 to 9 hours. If the sleeping period had been curtailed the test was not given. If something had been eaten just before going to bed or if the student had smoked just before coming in, the test was not given that day.

Few of the students drank much coffee so this was not considered a factor of great importance. Most of the students ate in the college dining hall and a separate study of food intake was made on a group of students to see if it might

[1] The temperature average 1932 to 1937 was 48.47°F.

have any bearing on our average rate.[2] The women were tested at any time during the intra-menstrual period.

Information concerning the activities of the students during the preceding day or days was recorded. The pulse and body temperature were always recorded, and after the spring

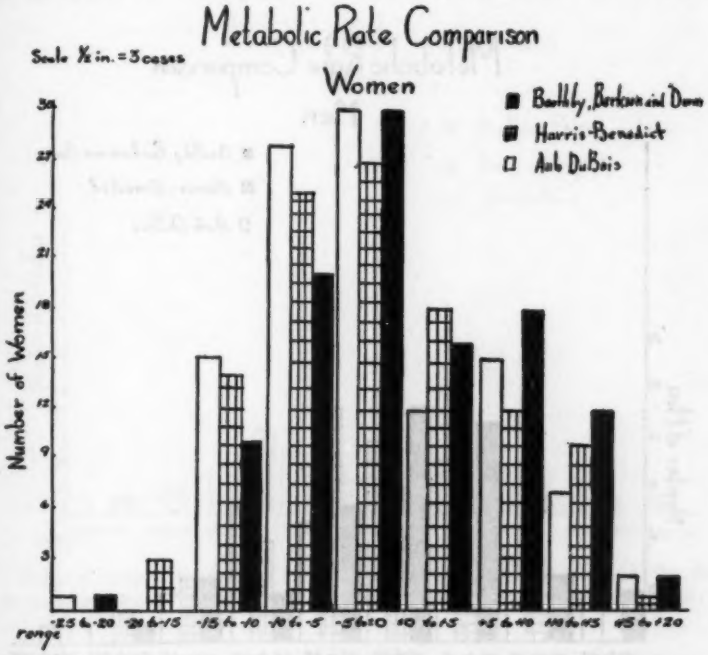

Fig. 1 Comparison of basal metabolic rates of women as calculated by the methods of: Boothby, Berkson and Dunn—Harris-Benedict—and Aub-DuBois.

of 1936 when a Baumanometer became available, blood pressure was determined.

During one winter the apparatus was taken to a room in the infirmary. The student to whom the test was given came in early, went to bed between 9 and 10 o'clock and was given the test at 7 o'clock the next morning without dressing

[2] Table not given in this paper.

or going further than the next room. As all students who came to the infirmary were carefully supervised it was possible to determine whether they were in true basal state.

A series of tests made in this way convinced us that they were in a true basal state and that our rest period in the basal metabolism room had been adequate. Variations in

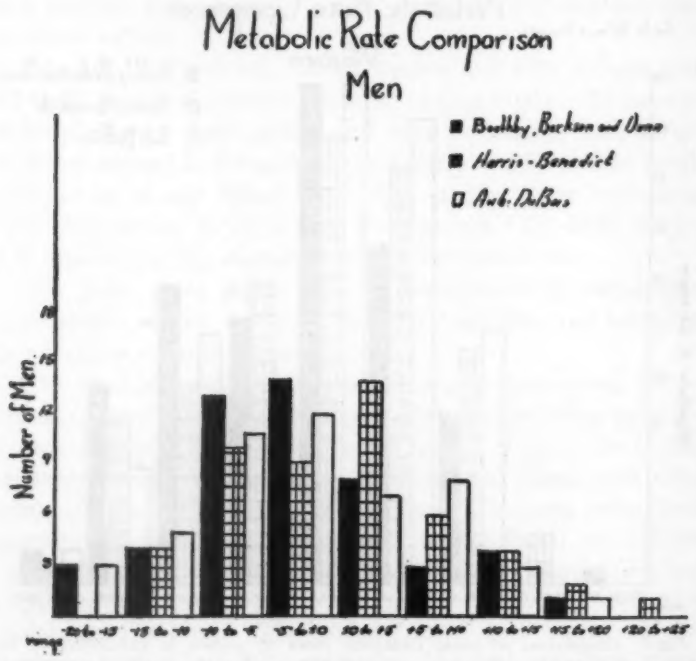

Fig. 2 Comparison of basal metabolic rates of men as calculated by the methods of: Boothby, Berkson and Dunn—Harris-Benedict—and Aub-DuBois.

basal rate of students given tests in both the infirmary and the dormitory basal metabolism rooms were insignificant. Day to day variations in basal rate when given in the same room were often greater.

Tests were given on 3 or more different days except in cases where tests on 2 different days were very nearly alike.

Early in our study two or more 6-minute tests were given each time the student came. It was seldom that there was much variation in consecutive tests given on the same day, so that this practice was discontinued except for a preliminary test the first time that a student came.

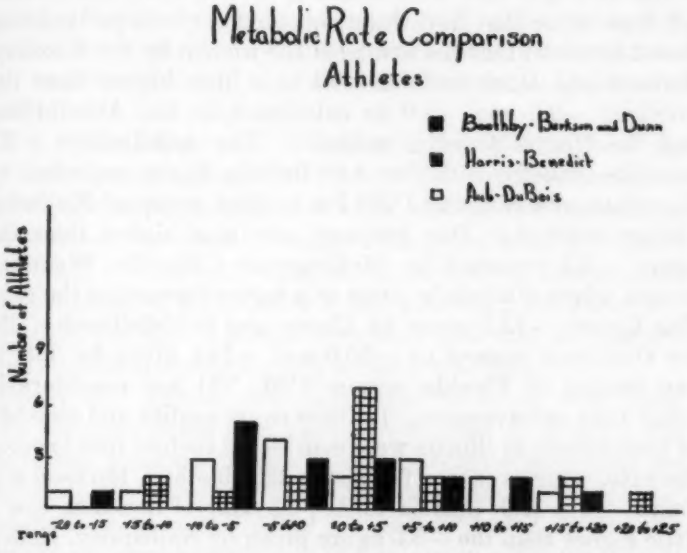

Fig. 3 Comparison of basal metabolic rates of athletes as calculated by the methods of: Boothby, Berkson and Dunn—Harris-Benedict—and Aub-DuBois.

RESULTS

The basal rates were calculated by the methods of 1) Boothby, Berkson and Dunn '36), 2) Harris-Benedict, and 3) Aub-DuBois, modified by Boothby and Sandiford.

Figures 1 and 2 are comparison graphs showing the differences in basal rate as calculated by the three above-mentioned methods. Figure 3 is that of athletes.

In spite of the very wide variation in range of basal rate the average rate for women is —0.4 by Boothby, Berkson and Dunn method; —1.9 Harris-Benedict, and —2.3 Aub-Dubois,

with probable errors of ±0.47 to ±0.52. The corresponding averages for men were −2.5, +0.8 and −1.6 respectively, with probable errors of ±0.74 to ±0.78.

DISCUSSION

The averages that were obtained in this study are nearer ±0 than some that have been reported for college students. It will be noted that the averages for women by the Boothby, Berkson and Dunn method −0.4 is a little higher than the averages −2.3 and −1.9 as calculated by the Aub-DuBois and the Harris-Benedict methods. The Aub-DuBois −2.3 coincides exactly with the Aub-DuBois figure reported by Gustafson and Benedict ('28) for a select group of Wellesley College students. Our averages are also higher than the figure −3.1 reported by McKetterick ('36) for Wyoming women where altitude is given as a factor increasing the rate. The figures −13.2 given by Coons and Schiefelbusch ('32) for Oklahoma women or −10.6 and −14.1 given by Tilt in two studies of Florida women ('30, '35) are considerably lower than our averages. In these cases quality and quantity of food as well as climate were considered factors that lowered the rate. Our averages for men −2.5 Boothby, Berkson and Dunn, +1.6 Aub-DuBois and +0.8 Harris-Benedict are a little higher than the −5.6 figure given by Nalbandov, Heller, Krause and Purdy ('38) in a study of Oklahoma college men.

In the case of the men students the average rate as calculated by the Boothby, Berkson and Dunn method is slightly lower than the Aub-DuBois and the Harris-Benedict methods.

In general it would appear that the Boothby, Berkson and Dunn method gives more normal curves for basal rates. The irregularities on curves for seventeen athletes are so similar to those for the larger group of fifty men that it seems probable that the athletes are responsible for these irregularities. Two groups of men as large as the group of women would probably be more satisfactory although the probable error in average rates for men is only ±0.74 to ±0.78.

The great length of range in basal rate of students who were considered normal in every way seemed to call for some explanation. Since we had carefully excluded any who were considered abnormal one might question whether students showing a wide range of basal rate were in true basal state during the tests. Those who showed the greatest range in basal rate usually averaged well within the ±10.0. Usually the student whose rate seemed much too low or too high was unconscious of any emotional disturbance and seemed to us to be in true basal state.

W.B. a freshman gave a high rate of +18 at first but gradually it diminished to +4.1. At this time we found out that the swimming season had ended and that he was getting less exercise than usual. A.C. on the other hand gave such uniformity on two tests that the third seemed unnecessary. One test came on a day after a ball game and the other in a non-active period. All who knew him said that he was unusually uniform in studies and athletic activities.

Among the women, J.W. appeared to be in true basal state, but gave three plus rates, the highest of +28.7. She always had a normal pulse and blood pressure, and when we sent her to the college physician, he found her as on all other examinations, normal. After a vacation period of 10 days in which she slept and ate well the physician gave her a basal metabolism test. We also gave another test 2 days later. Both of these tests were near ±0. She finally admitted that up until the vacation period she had been working hard and that perhaps had thought considerable about her studies and social activities. She said that she was not conscious of worrying but perhaps felt a bit let down when examinations and sorority elections were over.

J.L. was a student who was given the tests during a summer session when he was doing hard physical work in the college dairy department. His muscles were well developed and his slightly high rate was probably due to the effect of overexercise.

Somewhat older people, members of the faculty and faculty wives who came to us for tests, rarely showed a great variation in basal rate. Often the variation in a series of three to five tests over a period of several weeks or months was only a few per cent. It seemed probable that the more settled or normal manner of living may account for the more uniform basal rate of this older group.

G. W. Henry ('30) says that elated and overactive states correlate with the high basal rate and that depressed states correlate with low rates. Henry suggests the probability that some of the normal variation in basal rate is due to the emotional state of the individual tested. It seems probable that the wide range or variation in basal rate of college students together with overactivity in some cases may be explained on this basis.

The average weight of the students tested was relatively high. Forty-five to 50% of both men and women are within 5% of the normal range.[3] The food intake seemed adequate in calories, vitamins and minerals, and the protein was 15%, or it was 4% higher than that reported by Coons and Schiefelbusch ('32) for Oklahoma women.

SUMMARY

The average basal rate of the Connecticut State College women was found to be —2.3 Aub-DuBois; —1.9 Harris-Benedict, and —0.4 Boothby, Berkson and Dunn. The average for men students was —1.6 Aub-DuBois; +0.8 Harris-Benedict, and —2.5 for Boothby, Berkson and Dunn. For an individual, ranges in rate may be of as much or more physiological significance than average rate.

The excellent quality and quantity of food may be a factor in producing a relatively high average, and the extremes of range may correlate with overactivity or with emotional states common to college life.

[3] From the records of Dr. Ralph L. Gilman, resident physician Connecticut State College, Storrs, Connecticut.

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